Plant Decision-Making in the Arbuscular Mycorrhizal Symbiosis: the Role of Spatial Structure, Nutrient Availability and Partner Identity

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Alicia Argüello García

aus

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Promotionskomitee:

Prof. Dr. Bernhard Schmid (Leitung und Vorsitz)
Prof. Dr. Andres Wiemken
Prof. Dr. Marcel van der Heijden
PD. Dr. Lindsay Turnbull

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Promotionskomitee:
Prof. Dr. Bernhard Schmid (Vorsitz)
Prof. Dr. Andres Wiemken
Prof. Dr. Marcel van der Heijden
PD. Dr. Lindsay Turnbull

This thesis is dedicated to the memory of my father, Florentino Argüello Sierra.

Porque desde niña me motivaste a superarme cada día
pero sin olvidar jamás quién soy ni de dónde vengo.

Contents

General Introduction

Recently, some researchers have called for a new field of plant biological research, that of plant 'neurobiology' (Brenner *et al.,* 2006; Baluška,Volkmann & Menzel, 2005), where plants would have "brain-like" control (Hodge, 2009) over its responses. These parallels between animal and plant behaviour were first proposed by Charles Darwin in the 19th century (Trewawas, 2005, 2007). This is however, a controversial field where detractors have argued that plant "neurobiology" adds little to what might be explained with existing areas of plant biology (Alpi *et al.,* 2007; Struik, Yin & Meinke, 2008).

In contrast to animals, plants have no central nervous system with which to integrate decision-making when foraging for resources. Nevertheless, integration of information must be important for plants. This is particularly evident in the case of symbioses such as the mycorrhizae, where the plant invests photosynthesized carbon into a fungal partner in exchange for access to mineral nutrients foraged by the fungus. Furthermore, because the mycorrhizal mycelia colonize several individuals at the same time, plant parts are connected in a network without hierarchy similar to workers in an ant colony (Greene & Gordon, 2007). Hence, in nature, plants and mycorrhizal fungi commonly form large networks (Whitfield, 2007), where different individuals of plants are interconnected by mycorrhizal hyphae in which nutrients and carbon can move from one plant to another (Selosse *et al.,* 2006). This creates many opportunities for the exchange of resources that may lead to positive or negative interactions among species. Theoretically, selection should favour individuals that take more than they give, but two closely interacting species of plant and fungus may evolve a mutualistic relationship if cheaters can be excluded (Egger & Hibbett, 2004; Kiers & van der Heijden, 2006). The existence of this range from negative to positive interactions between mycorrhizal fungi and plants and its potential variation under environmental change are of

particular interest in this thesis, especially with regard to plant decision making, partner selection and the net outcome of the plant–AMF symbiosis.

Mycorrhizal symbiosis

Mycorrhizae are the most prevalent symbiotic association found in the plant kingdom being present in 80% of plant species (Simon *et al.*, 1993; Schüβler *et al.*, 2001). Mycorrhizal symbiosis evolved very early in the evolution of plants, when the plants moved onto dry land. The first land plants had no real roots and therefore the acquisition of nutrients was probably a major challenge to plants (Smith & Read, 2008). In soils, diffusion of phosphate is slow, and depletion zones rapidly build up around the surface of absorbing roots. Because of hyphae, the mycorrhizal fungal partner can extend outside this nutrient depletion zone of the rhizoshpere and acquire phosphate from a larger volume of soil. Although it is unknown how the symbiosis first began, the fungus may have initially been a pathogen, but the association evolved to be beneficial for both: the plant receiving phosphate and other resources and the fungus obtaining carbon from the host plant (Hodge, 2009) (Fig.1). This symbiosis is often considered mutualistic because mycorrhizal fungi receive carbon from the plant, however the net effect on plant fitness ranges from mutualistic to parasitic (Kiers & van der Heijden, 2006), depending on the ecological conditions and plant–fungus combinations.

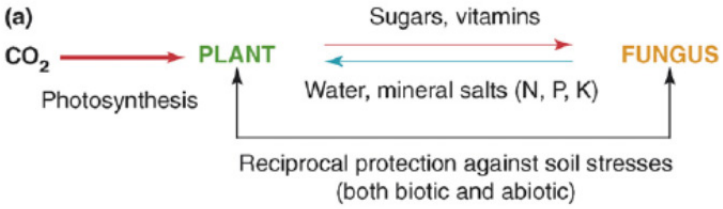

Figure 1. Resource transfers between a plant and its mycorrhizal fungal partners (Selosse *et al.* 2006).

There are seven different types of mycorrhizal symbiosis that can be established depending on the host plant–fungal combination (Smith & Read, 2008). Plants most

commonly associate with arbuscular mycorrhizal fungi (AMF). These are named after the 'arbuscule' (meaning 'little tree', Fig. 2), a fungal structure that forms in the roots of the host and is the site of phosphate transfer between the AMF and the host plant.

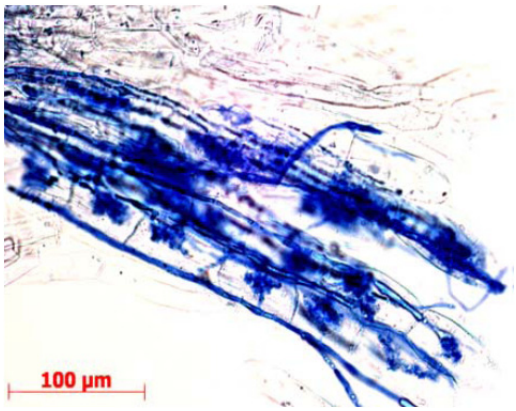

Figure 2. Root of *P. lanceolata* colonized with AMF. Tree–like structures are arbuscules.

Traditionally, because of the low number of described AMF species (based on morphological traits only around 200 AMF species have been described (Schüßler & Walker, 2010)), it has been assumed that all AMF could colonize and provide a similar function to any plant host. AMF do associate with the majority of plants within a community, but the amount of soil nutrients transferred to the plant greatly depends on the identity of the AMF species present (van der Heijden *et al.*, 2003) (Fig 3). Consequently, changes in the mycorrhizal composition in a plant community may influence biomass, nutrient status and the relative abundance of plant species (Girlanda *et al.*, 2006; Bever, 2002). A comparison by van der Heijden (2003) with other biotic factors such as insect herbivores, pathogens and fungal endophytes (Bentley & Whittaker, 1979; Windle & Franz, 1979; Cottam, 1986; Paul, 1989; Clay *et al.*, 1993) indicated that the impact of different AMF species on the coexistence of plant species is comparable to the impact of those biotic factors.

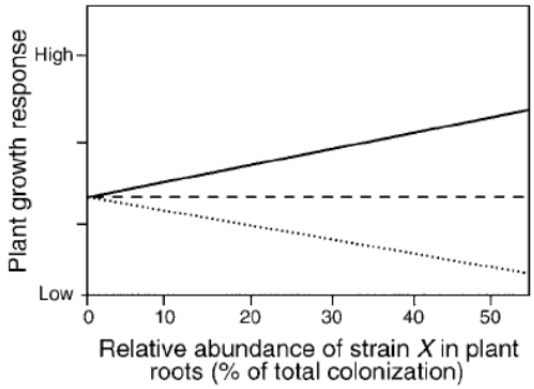

Figure 3. Three possible relationships between relative abundance of AMF types in roots and plant growth response from Kiers and van der Heijden, 2006: (1) the solid line indicates a positive relationship between abundance of a particular AMF type and benefits to plant growth; (2) the dotted line indicates a negative relationship; (3) the dashed line indicates that no relationship exists between the abundance of an AMF type and its impact upon plant growth.

However, despite the proven ecological relevance of AMF, there are still many gaps in our understanding of the AMF symbiosis. Most of these gaps concerns to the role of AMF foraging (e.g. the amount of nutrients that AMF take up and deliver to the plant) and its variation depending on partner identity (both AMF and plant) and the environmental conditions (e.g. nutrient availability).

Plant decision-making: resource allocation

The allocation of resources to above– and belowground biomass is an important decision for plants and impacts nutrient capture, reproduction, fitness and competitive ability (Hodge, 2009). There is evidence, about how the environmental conditions influence this resource allocation; for example, plants allocate biomass to acquire a limiting resource such light with increased leaf aread or nutrients or water with increased root length (Fig. 4). Under low nitrogen (N) availability, plants allocate more resources to their roots to enhance N capture (Reynolds & D'Antonio, 1996). Moreover, Campbell *et al.* (1991) showed that plants are able to spatially specifically allocate resources *within* the root system (Fig. 4). Thus, plants

growing in patchy environments may identify and explicitly allocate resources to proliferate new roots in high nutrient patches.

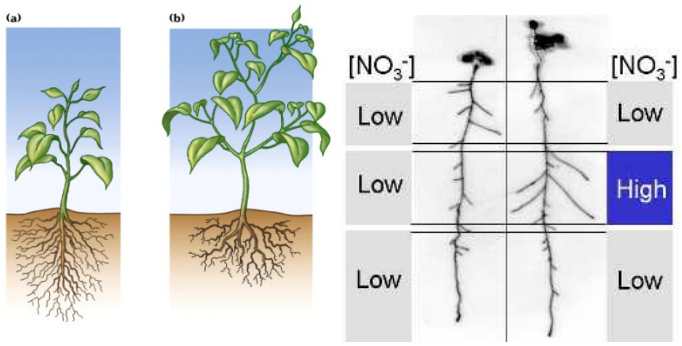

Figure 4. Adjustment of root-to-shoot ratio when soil nutrients are scarce (left panel). The plant in (a) is growing in limiting soil nutrients and thus allocates more carbon to root growth, while the plant in (b) allocates more to the above ground shoot under plentiful nutrients. **Change in biomass allocation within root system (right panel).** The plant on the right is growing in a patchy nutrient environment and thus allocates more carbon to the nutrient-rich patch in the middle.

Root proliferation is also observed when mycorrhizal hyphae are present in patches (Hodge, 2001a, b). However, there are risks for the plant in relying on the AMF to acquire the nutrients, i.e. the fungus may acquire the nutrients for itself or pass them to another plant through mycorrhizal networks (Hodge, 2009). Root proliferation often does take considerable time to occur (Hodge *et al.,* 2000), and that delay may be a means for the plant of testing if the nutrient patch is durable or not, which could be seen as an "intelligent response" (Trewavas, 2006).

It has also been suggested that AMF may use this root proliferation mechanism in nutrient-rich patches as a means to obtain carbon from their host (Fitter, 2006). The host detects increased phosphate ratios at the site of the fungal arbuscule and allocates carbon to that area of the root. Instead of being used to construct new roots (as in the proliferation response caused by the nutrient increase), the carbon is acquired by the AMF. If the phosphate signal continues or increases, then the flow of carbon becomes so intense that root

proliferation begins (Fitter, 2006). This root–AMF interaction might explain how and why AMF hyphae inside the root manage to acquire carbon without 'cheating' on their host (Hodge, 2009).

Plant decision-making and AMF symbiosis

As previously mentioned, plants are known to be able to respond to environmental factors such as nutrient availability, and extending that concept to plant responses in the presence of AMF partners is not such an improbable assumption. Plants can likely modify their allocation to AMF partners depending on the specific abilities of the AMF to provide nutrients or other resources. Moreover, plants are likely to invest less carbon in AMF, regardless of AMF identity, if plant growth is carbon limited rather than nutrient limited (Fig. 5). Economic models applied to mutualisms (e.g. rhizobial bacteria or mycorrhizal fungi) also suggest that mutualisms decline with increasing nutrient availability (Schwartz and Hoeksema, 1998; Johnson, 1993; Corkidi *et al.*, 2002). Therefore, AMF are less beneficial to plants when resources are abundant.

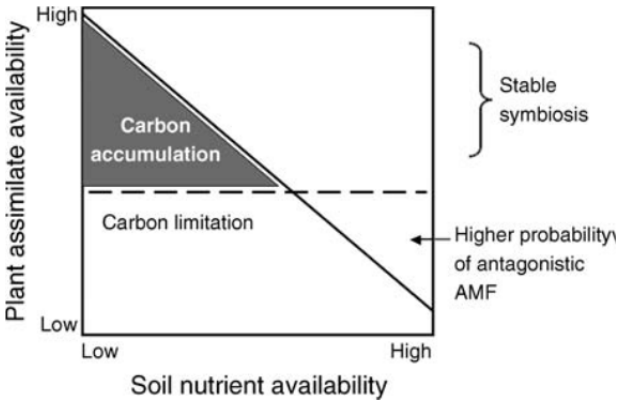

Figure 5. Hypothetical model from Kiers and van der Heijden (2006) showing plant assimilate availability as a function of nutrient availability (solid line). The dashed line shows the amount of carbon required for growth, maintenance, and respiration. At low nutrient availability, carbon will tend to accumulate, while, at high nutrient availability, carbon is a limiting resource. The symbiosis between plants and AMF is predicted to be most stable under surplus C supplies. At high nutrient and low C availability, there is a higher probability of antagonistic AMF relationships.

Substantial host-specificity of AMF with plants has been demonstrated in a number of temperate grasslands (Bever *et al.*, 1996; van der Heijden *et al.*, 1998a). This host-specificity, in combination with AMF diversity, promotes the maintenance of plant community diversity (van der Heijden *et al.*, 1998b; Bever *et al.*, 2001; van der Heijden, 2002). Furthermore, differences in AMF communities have been found among plant species, ecosystems and seasons (Bever *et al.*, 2001; Husband *et al.*, 2002a,b; Öpik *et al.*, 2006) and even between different parts of the same root system (e.g. roots and root nodules of legumes, Scheublin *et al.*, 2004).

In fact, legumes have already shown signs of partner choice by the practice of "host sanctions" (Denison, 2000) against less-effective rhizobial strains. As in the case of legumes, host plants are typically infected by multiple AMF (Vandenkoornhuyse *et al.,* 2002; Scheublin *et al.,* 2004) and under conditions of multiple infections, enforcement mechanisms against poor-quality partners are particularly important in stabilizing cooperation (Kiers *et al.*, 2006). Recently, preferential allocation of photosynthate by plants to the more mutualistic AMF was confirmed (Bever *et al.*, 2009; Kiers *et al.*, 2011). This preferential allocation in which plants control and therefore "decide" where to allocate resources at the scale of a root system or rootlet might enhance the success of the soil patches with greater abundance of beneficial fungi (Bever *et al.*, 2009). Moreover, a recent study demonstrated that not only did the plant host reward greater carbon to the more cooperative AMF but also the AMF provided more phosphorous to those roots contributing more carbon (Kiers *et al.*, 2011). In this case the plant–AMF symbiosis, in contrast to many other mutualistic associations, acts as a biological market (Noë and Hammerstein, 1995; Schwartz and Hoeksema, 1998), where both partners control the exchange of resources (usually defined as trade) and consequently, the performance of the symbiosis. Nevertheless, experimental support (Bever *et al.*, 2009; Kiers *et al.*, 2011; Verbruggen *et al.*, 2012) for partner choice in contributing to the maintenance of

the mutualism in the plant–AMF symbiosis is minimal with no study to our knowledge, reporting the existence of a bidirectional partner control *in vivo*.

Concept and outline of this thesis

In my thesis, I explore the complex plant–AMF symbiosis and link it with the idea of plant decision-making. I had two general aims: (1) to investigate interactions under different environmental conditions (e.g. nutrient availability) among several species of both AMF and plants and (2) to further understand the resource exchange (trade) between partners of the plant–AMF symbiosis. In order to address these issues, I employed several experimental approaches including analyses of plant growth, nutrient concentrations of plant tissues and resource exchange between plant and AMF using the radioactive isotopes ^{33}P and ^{14}C. I also assessed preferential allocation to the AMF by measuring mycorrhizal abundance (percentage of root colonized by AMF), relative root biomass and carbon allocation. Furthermore, I developed split-root systems to examine the ability of plant species to perceive and integrate information of heterogeneous biotic (e.g. variation of AMF presence or identity) and abiotic (e.g. variation in nutrient availability) environments. This thesis aims to contribute to our knowledge of the existence of "decision-making" processes in plants and to elucidate the conditions of trade determining the outcome of the plant–AMF symbiosis.

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Summary

This thesis investigated decision-making processes in plants associated with Arbuscular Mycorrhizal Fungi (AMF) and the conditions controlling the trade and net outcome of the symbiosis. I found evidence that plants can integrate information from heterogeneous biotic and abiotic environments and modify their responses accordingly. Moreover, I demonstrate preferential allocation and reciprocal rewards as potential mechanisms for the persistence of mutualism in the plant–AMF symbiosis.

Chapter 1 compiles data from a large experimental set-up with 56 different combinations of plant species and AMF. This study demonstrates plant x AMF species-specific responses of plant biomass and AMF colonization to the plant–AMF symbiosis. Compared with AMF-free control treatments plant biomass was increased or decreased by AMF, depending on the particular species combination. The results obtained in this experiment allowed me to select the *a priori* most suitable plant–AMF combinations for further experiments where I linked the concept of plant decision-making with the level of benefit obtained by the plant from the symbiosis with AMF.

In **Chapter 2** I used split-root systems to analyze the response of plant parts to homogeneous of heterogeneous AMF presence. The spatial structure in AMF presence was achieved by applying one of two AMF species or a non-mycorrhizal control treatment on each half of the split-root systems of a single plant of *Trifolium pratense.* The aim was to test if the plant could select between AMF species. Increased inoculation (no AMF < AMF in one half < AMF in both halves of the split-root system) of the supposedly more beneficial AMF species *(Diversispora celata)* enhanced plant biomass and shoot-root ratio. On the other hand, the full positive effect of the supposedly less beneficial AMF species *(Funneliformis mosseae)* was already reached when only one half of the split-root system was inoculated. As predicted,

single plants in heterogeneous AMF combinations preferentially allocated root biomass to the side with AMF or (if both sides had AMF) to the more beneficial AMF (*D. celata*).

In **Chapter 3** and using again split-root systems, I examined the effects of co-occurring interactions among plant species identity, AMF presence and different levels of nutrient availability. I tested plant responses at the level of the entire plant as biomass and nitrogen accumulation and as biomass allocation, and at the level of the two halves of the root system as belowground biomass. I found that in homogeneous treatments, *T. pratense* was mostly and positively affected by AMF presence. *Plantago lanceolata* was equally and additively affected by AMF presence and nutrient availability for biomass, but negatively affected by AMF presence for nitrogen content. In heterogeneous treatments *P. lanceolata* showed positive signs of integration for nutrient heterogeneity, i.e. increased root biomass allocation to nutrient-rich patches. In addition, *P. lanceolata* showed positive integration for AMF heterogeneity at low nutrient availability, whereas *T. pratense* showed no integration for nutrient heterogeneity and negative integration of AMF heterogeneity. My results show correlative responses between the two halves of root systems exposed to spatial heterogeneity in AMF presence or nutrient availability. This suggests that plants can integrate signals coming from different parts of the root system. In particular, biomass allocation towards roots in AMF- or nutrient-rich patches was increased at the within-plant level, which was in contrast to the decreased allocation of biomass to roots when entire plants were growing in homogeneous AMF or nutrient conditions. This study suggests that plants perceive AMF presence and nutrient availability as different signals and are able to show integrated responses accordingly.

In **chapter 4** I tested the hypotheses that preferential allocation to the more beneficial AMF and reciprocal rewards between partners are both processes that stabilize cooperation in the plant–AMF symbiosis. I combined the use of radioactive isotopes (^{14}C and ^{33}P) and

compartmentalization of model systems, to examine *in vivo* the exchange of resources between plants and AMF. I used two plant species differing in their mycorrhizal dependencies (*P. lanceolata* and *T. pratense*) and two different AMF isolates varying in their beneficial effects on plants (*Rhizophagus irregularis* and *F. mosseae*). This set-up allowed me to examine variation in the plant–AMF trade in near-natural conditions. I confirmed higher allocation to the more cooperative AMF (*R. irregularis*) and, in treatments where both AMF were present, reduced allocation to the less cooperative AMF (*F. mosseae*). AMF partners seemed to be better symbionts for *T. pratense* than for *P. lanceolata*, but the former also allocated relatively more carbon to the AMF partners than did the latter. Additionally, I proved that the carbon costs per unit of phosphorous transferred to the plant were always higher for *P. lanceolata* than for *T. pratense*, and especially when associated with the less cooperative *F. mosseae*. Although further studies across a greater diversity of symbionts and environments are needed, my study shows that reciprocal rewards from both partners *in vivo* enhanced the mutualism and might explain the evolutionary persistence of the plant–AMF symbiosis.

Zusammenfassung

Diese Dissertation untersuchte sowohl Entscheidungsprozesse in Pflanzen, die mit arbuskulären Mykorrhizenpilzen (AMF) assoziiert sind, als auch die Bedingungen, welche den Leistungsaustausch und Ergebnis dieser Symbiose kontrollieren. Ich konnte belegen, dass Pflanzen Informationen von heterogenen biotischen und abiotischen Umgebungen integrieren können, und ihre Reaktionen entsprechend anpassen können. Darüberhinaus zeigte ich, dass bevorzugte Allokation und reziproke Gegenleistungen mögliche Mechanismen sind, welche die Beständigkeit von Mutualismus in Pflanzen-AMF Symbiosen erklären können.

Kapitel 1 fast einen umfassenden Datensatz aus einem grossen experimentellen Versuchsaufbau zusammen, welcher aus 56 verschiedene Kombinationen von Pflanzen und arbuskulären Mykorrhizenpilzen (AMF) besteht. Die Studie zeigte Pflanzen x AMF artspezifische Antworten von Pflanzenbiomasse und AMF-Kolonisierung auf die Pflanzen-AMF Symbiose. Verglichen mit AMF-freien Kontrollbehandlungen wurde die Pflanzenbiomasse durch AMF erhöht oder erniedrigt, je nach spezifischen Artkombinationen. Die in dem Experiment gewonnenen Resultate erlaubten mir die Auswahl einer "a priori" bestmöglichen AMF-Pflanzenkombination für weitere Experimente, worin ich das Konzept von Entscheidungsprozessen in Pflanzen mit dem Grad des Nutzens, den eine Pflanze aus der Symbiose mit AMF erzielt, verband.

In **Kapitel 2** benutzte ich Wurzelteilungssysteme um den Einfluss von homogener oder heterogener Präsenz von AMF auf Pflanzenbestandteile zu analysieren. Die räumliche Struktur der Präsenz von AMF wurde erzielt, indem eine von zwei AMF Arten oder eine nicht-mykorrhizale Kontrollbehandlung in jeder

Hälfte des Wurzelteilungssystems von einzelnen *Trifolium pratense* Testpflanzen ausgebracht wurde. Ich fand, dass eine zunehmende Inokulation (kein AMF < AMF in einer Hälfte < AMF in beiden Hälften des Wurzelteilungssystems) der vermeintlich günstigeren AMF Art (*Diversispora celata)* die Pflanzenbiomasse und das Spross-Wurzel-Verhältnis erhöhte. Dahingegen war der vollständige positive Effekt der vermeintlich weniger günstigen AMF Art (*Funneliformes mosseae*) bereits dann erreicht, wenn bloss eine Hälfte des Wurzelteilungssystems inokuliert war. Einzelpflanzen in heterogenen AMF-Kombinationen teilten - wie vorausgesagt - der Seite mehr Wurzelbiomasse zu, welche entweder AMF enthielt, oder (falls beide Seiten AMF enthielten) derjenigen, welche die günstigere AMF Art enthielt.

In **Kapitel 3** benutzte ich wieder Wurzelteilungssysteme und untersuchte den Effekt von gleichzeitig auftretenden Interaktionen zwischen Pflanzenidentität, Präsenz von AMF und verschiedenen Stufen von Nährstoffverfügbarkeit. Ich testete die Reaktion von Pflanzen auf Ebene der gesamten Pflanze sowohl gemessen an Biomasse und Stickstoffakkumulation, als auch an Biomasse-Allokation - und auf Ebene der zwei Hälften des Wurzelsystems, gemessen an unterirdischer Biomasse. Ich fand, dass in homogenen Behandlungen *T. pratense* am stärksten und positiv von AMF Präsenz beeinflusst wurde. Die Biomasse von *Plantago lanceolata* wurde hingegen gleichermaßen und additiv von AMF Präsenz und Nährstoffverfügbarkeit beeinflusst, dessen Stickstoffanteil sogar negativ.

In heterogenen Behandlungen zeigte *P. lanceolata* vermehrte Anzeichen von Integration bei Nährstoffheterogeneität, d.h. erhöhte Allokation von Wurzelbiomasse an nährstoffreiche Stellen. Zusätzlich zeigte *P. lanceolata* erhöhte Integration bei AMF Heterogeneität und niedriger Nährstoffverfügbarkeit, *T. pratense* hingegen zeigte keine Integrationseffekte beiNährstoffheterogeneität und reduzierte Integration

bei AMF Heterogeneität. Meine Resultate deuten auf korrelative Antworten zwischen den zwei Hälften des Wurzelsystems einer einzelnen Pflanze hin, welche räumlicher Heterogeneität von AMF oder von Nährstoffverfügbarkeit ausgesetzt sind. Dies deutet darau hin, dass Pflanzen Signale von verschiedenen Teilen integrieren können. Besonders die Allokation von Biomasse zu den Wurzeln in AMF- oder nährstoffreichen Stellen war erhöht – im Gegensatz zu der reduzierten Allokation von Biomasse zu den Wurzeln wenn ganze Pflanzen in homogenen AMF oder -nährstoffreichen Bedingungen wuchsen. Diese Studie weist darauf hin, dass Pflanzen die Präsenz von AMF und Nährstoffen als verschiedene Signale wahrnehmen, und dass sie fähig sind, integrativ darauf zu reagieren.

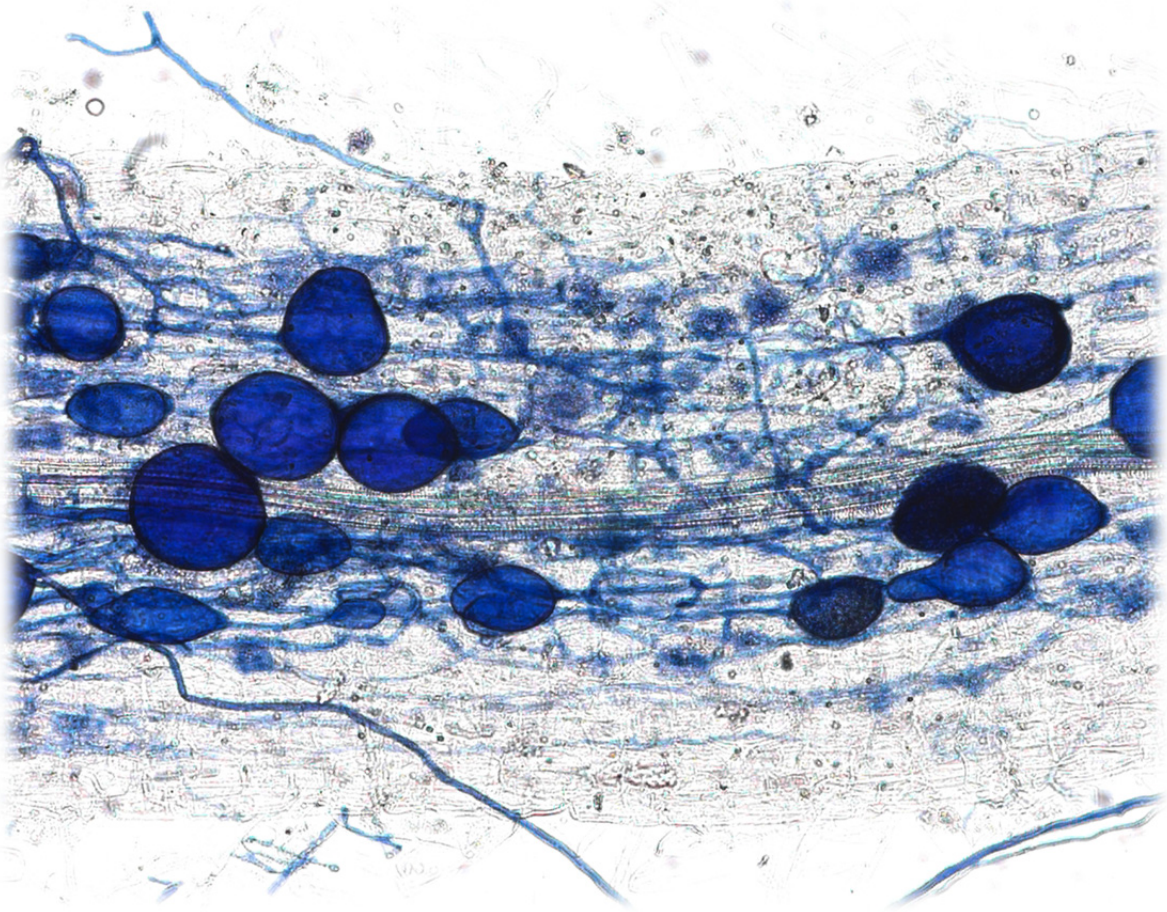
In **Kapitel 4** testete ich die Hypothese, dass bevorzugte Allokation zu günstigeren AMF als auch reziproke Gegenleistungen zwischen Partnern Prozesse darstellen, welche die Kooperation in Pflanzen-AMF-Symbiosen stabilisieren. Ich kombinierte radioaktive Isotopenmethoden (^{14}C und ^{33}P) und Kompartimentierung von Modellsystemen, um den *in vivo* Austausch von Ressourcen zwischen Pflanzen und AMF zu untersuchen. Ich benutzte zwei Pflanzenarten, die sich in ihren mykorrhizalen Abhängigkeiten unterscheiden (*P. lanceolata* und *T. pratense*), und zwei verschiedene AMF Isolate, die verschieden positive Effekte auf Pflanzen zeigen (*Rhizophagus irregularis* und *F. mosseae*). Dies erlaubte mir, Variation im Pflanzen-AMF Austausch unter naturnahen Bedingungen zu untersuchen. Ich bestätigte eine höhere Allokation zu der kooperativeren AMF Art (*R. irregularis*) und, bei Behandlungen mit beiden AMF, geringere Allokation zu der weniger kooperativen AMF Art (*F. mosseae*). AMF Partner schienen für *T. pratense* Symbionten von höherer Qualität zu sein als für *P. lanceolata*. Umgekehrt stellte *T. pratense* auch relativ mehr Kohlenstoff für die AMF Partner bereit als *P. lanceolata*. Zusätzlich

konnte ich beweisen, dass die Kohlenstoffkosten pro Einheit Phosphor, welche auf die Pflanzen transferiert wurden, für *P. lanceolata* immer höher waren als für *T. Pratense*insbesondere in der Assoziation mit dem weniger kooperativen *F. mosseae*. Obwohl weitere Studien über eine grössere Diversität von Symbionten und Umweltbedingungen nötig sind, zeigt meine Studie bereits, dass reziproke Gegenleistungen von beiden Partnern *in vivo* den Mutualismus verstärken, was die evolutionäre Beständigkeit von Pflanzen-AMF Symbiosen erklären könnte.

Chapter One

Interactive effects in the plant–AMF symbiosis: partner identity matters

Alicia Argüello, Marcel van der Heijden, Andres Wiemken and Bernhard Schmid



Abstract

Symbioses between plants and arbuscular mycorrhizal fungi (AMF) consist of intimate long-term associations in which the host plant obtains nutrients and other benefits in return of photosynthates. In spite of such apparent bi-directional cooperation, the effects that AMF have on plants can range from highly positive to negative depending on the identity of both partners and on the environmental conditions. At present, however, the mechanisms that affect the interactions between AMF and plants still remain unknown despite being of obvious importance to ecosystem processes. The aim of this study was to analyze the range of interactions and responses among seven plant species (including a non-mycorrhizal one) and seven AMF isolates plus a non-mycorrhizal control. We found large differences in plant responses (i.e. total plant biomass) and AMF colonization rates among 56 different AMF-by-plant treatment combinations, which could not be predicted by adding main effects of AMF and plant species. Significant interactions between these two factors indicated that specific AMF isolates were beneficial to some plant species, in particular *Trifolium pratense,* and detrimental to others, in particular *Plantago lanceolata,* whereas other AMF isolates showed exactly the opposite pattern. This demonstrates that plant–AMF associations can be specific, indicating isolate-by-species specialization and co-adaptation similar to other associations of plants with pollinating, consumer or seed dispersing animals. However, it remains to be explored what ecological or evolutionary correlates the occurrence of specialists vs. generalists on either side of the plant–AMF associations might have and what its consequences for community assembly and ecosystem functioning might be.

Key words: AMF–plant association, co-adaptation, interactions, specialization, symbiosis.

Introduction

Interactions among species are one of the main drivers for the diversification and organization of life (Thompson, 1999). In ecology many studies have analyzed the structure and characteristics of these interactions (Thebault & Fontaine, 2008), such as the occurrence of specialists and generalists on the different side of the interaction, to identify common features and deduce general mechanisms responsible for community assembly and ecosystem functioning (Martinez, 1994; Thébault & Loreau, 2005). In nature, species interact to obtain resources such as nutrients, energy (in the form of light or organic matter) or space. These interactions may lead to competition or symbiosis between species. Symbioses consist of intimate long-term associations in which both organisms cooperate. However, sometimes this cooperation results in one partner benefiting more than the other such that a wider definition of symbiosis includes even cases where one partner benefits at the expense of the other (Johnson *et al.*, 1997).

One of the most widespread symbioses on Earth is the mycorrhizal symbiosis between fungi and plant roots, estimated to be approximately 400 million years old (Simon *et al.*, 1993; Remy, 1994). An important group of symbiotic fungi are the arbuscular mycorrhizal fungi (AMF) belonging to the phylum Glomeromycota. The symbiosis between AMF and terrestrial plants is widespread in most of the world's ecosystems and is especially abundant in forbs, grasses and tropical trees; more than 60 percent of the known plant species can be colonized by AMFs (Trappe, 1987). In contrast to other mutualistic interactions, however, the association between plants and AMF so far has been considered rather unspecific because many different plant species can be colonized by single AMF isolates and a single plant species can host many different AMF isolates. There is correspondingly little knowledge about the specificity of plant–AMF associations and the potential causes leading to it as well as the potential consequences at the ecosystem level, such as community assembly and

ecosystem functioning (van der Heijden & Sanders, 2002). In the plant–AMF symbiosis, the plant provides assimilated carbon to the AMF which in return provides nutrients taken up from the soil to the plant (Koide, 1991; Jakobsen *et al.*, 1992; Govindarajulu *et al.*, 2005). Despite the generality of the plant–AMF association, there are some indications that specialization may nevertheless occur especially on the plant side of the interaction. For example, plant responsiveness to AMF can vary among plant species with different root morphologies (Baylis, 1975; Hetrick *et al.*, 1990; Smith, 1996). Plants with roots that are comparatively thick, with little branching and few or no root hairs are less effective in taking up water and nutrients from soil (Comas & Bouma, 2002) and therefore tend to show more positive growth responses to mycorrhizal colonization than plants with fine, highly branched roots and many root hairs. Furthermore, the effects of AMF on plants also depend on environmental conditions. Hence, such plants are thought to be more specialized and adapted to form associations with AMF. For example, when plants are colonized by AMF, the thin, long hyphae increase the capacity of taking up resources from the soil especially under low nutrient conditions.

There is less evidence for specialization on the side of the AMF for particular plant species. For example, single AMF individuals can colonize several plant individuals even of different species and thus establish a common mycorrhizal network (Simard & Durall, 2004) and a single plant individual or species can produce more biomass when it is colonized by several rather than a single AMF isolate (Wagg *et al.*, 2011). There can also be cases where the plant does not show a positive growth response to an AMF, suggesting that AMFs may sometimes be “cheaters” (Kiers & Van Der Heijden, 2006). There is, however, also the problem of defining taxonomic units for which specialization among different AMFs could be assessed. Based on morphological traits around 200 AMF species have been described (Schüßler & Walker, 2010) but based on sequence types there are many more isolates of AMF (Kivlin *et al.*, 2011). The question thus remains whether this large diversity of isolates is not

due at least in part to specific associations between particular AMF isolates and particular plant species. Some indication that specialization may also occur on the side of the AMF in plant–AMF associations is that AMF communities can vary between plant functional groups or species or even between parts of a root system (Helgason *et al.*, 2002; Bidartondo & Bruns, 2002; Vandenkoornhuyse *et al.*, 2003; Scheublin TR, Ridgway KP, Young JPW, 2004).

The aim of this study was to test for general and specific effects of the interaction between a range of AMF isolates and a range of plant species potentially co-occurring at grassland sites in Switzerland. We set up an experiment with seven plant species (including a non-mycorrhizal one) and seven AMF isolates plus a non-mycorrhizal control under controlled greenhouse conditions. We asked whether beneficial effects of AMF on plant growth could be predicted from the mean effect of an AMF isolate and the mean response of a plant species or whether specific plant–AMF associations deviated in positive or negative direction from these predictions. Such deviations would be expected if particular AMFs and plants would be mutually specialized or co-adapted to each other, similar to the mutualistic networks of plants with pollinators or seed-dispersing animals in the case of positive interactions (Jordano, 1987; Bascompte & Jordano, 2007) or to the antagonistic networks of plants and their consumers or pathogens in the case of negative interactions (Vacher *et al.*, 2008). Because several general patterns in the structure of mutualistic and antagonistic networks have been found (Bascompte *et al.*, 2003; Williams, 2011) we examined whether AMF and plants are as well "matched" in such a specific way. In such a case, as with other bipartite networks, losses of diversity on either side could have large consequences for community assembly and ecosystem functioning (Dunne & Williams, 2009).

Material and Methods

Experimental setup

The experiment was set up in the glasshouse of the Institute at the University of Zurich (43°23'N, 8°33'E, altitude 549m.a.s.l.) as a completely randomized design with two crossed factors, plant species x AMF isolate. There were seven plant species and seven AMF isolates plus a non-mycorrhizal control treatment, resulting in 56 treatment combinations. Each plant–AMF treatment combination was replicated four times resulting in a total of 224 pots.

Growth medium

We used a 1:9 volumes of a field-soil:quartz-sand mixture as growth medium. The field soil was collected from a natural grass–clover field at the agroscope Reckenholz research station in Zurich, Switzerland (47° 25'N, 8°31'E) and sieved through a 5 mm mesh before mixing. The mixture had a pH of 6.7 and was sterilized with gamma radiation at ca. 50 kGy (range 25–80 kGy LEONI, Aargau, Switzerland).

Biological material

We used seven different AMF isolates representing five fungal species (Table 1): ISCB 13 (*Funneliformis mosseae* (Krüger *et al.*, 2012) previously named *Glomus mosseae*), ISCB 39 (*Claroideoglomus etunicatum* (Krüger *et al.*, 2012) previously named *Glomus etunicatum*), ISCB 49 (*Claroideoglomus lamellosum* (Krüger *et al.*, 2012) previously named *Glomus lamellosum)*, ISCB 137 (*Rhizophagus irregularis* (Krüger *et al.*, 2012) previously named *Glomus intraradices*), BEG 75 (*Rhizophagus irregularis* (Krüger *et al.*, 2012) previously named *Glomus intraradices*), FACE 234 (*Diversispora celata* (Gamper *et al.*, 2009) previously named *Glomus eburneum*) and JJ 964 (*Funneliformis mosseae* (Krüger *et al.*, 2012) previously named *Glomus mosseae*). The first five were kindly provided by the

Botanical Institute of the University of Basel (Kurt Ineichen) and the last two by agroscope Reckenholz (Fritz Oehl).

Table 1. List of AMF isolates and species used in the experiment.

AMF isolate	AMF species	Author	N spores per gram
ISCB 13	*Funneliformes mosseae*	Krüger *et al.*(2012)	60
ISCB 39	*Claroideoglomus etunicatum*	Krüger *et al.*(2012)	90
ISCB 49	*Claroideoglomus lamellosum*	Krüger *et al.* (2012)	200
ISCB 137	*Rhizophagus irregularis*	Krüger *et al.* (2012)	55
BEG 75	*Rhizophagus irregularis*	Krüger *et al.* (2012)	50
FACE 234	*Diversispora celata*	Gamper *et al.* (2009)	15
JJ 964	*Funneliformes mosseae*	Krüger *et al.* (2012)	5

We used seven different plant species whose seeds were obtained from local suppliers (FENACO, Switzerland) or from the Botanical Institute of the University of Basel (Table 2). We pretreated the seeds in 5% chloride for ten minutes and thoroughly rinsed them four times with demineralized water. The seeds were then allowed to germinate in sterile sand and three germinated seedlings were transplanted into 800 ml pots. Dead seedlings were replaced within two weeks after initial transplanting.

Table 2. List of plant species used in this experiment.

Common name	Scientific name	Author	Type
Field wood-rush	*Luzula campestris*	(L.) DC.	Rush
Ribwort plantain	*Plantago lanceolata*	L.	Perennial weed
Flax	*Linum usitatissimum*	L.	Annual plant
Wild strawberry	*Fragaria vesca*	L.	Perennial weed
Bird's-foot trefoil	*Lotus corniculatus*	L.	Legume
Red clover	*Trifolium pratense*	L.	Legume
Wild garlic	*Allium vineale*	L.	Perennial weed

After this time we inoculated the plants with AMF. Since AMF isolates differed in the number of spores per gram soil (Table 1), we used different amounts of inoculum to add approximately 100 spores per seedling in each case, except where plants did not receive AMF.

Differences in amount of inoculum were corrected by adding a sterilized (by autoclaving for 60 min at 121 °C) mixture of the seven AMF isolates to avoid confounding effects due to different levels of nutrients added via the inoculation. The sterilized mixture was also used as inoculum for the non-mycorrhizal control treatment and to correct for differences in the amount of inoculum between isolates. In total, each pot received the same amount of inoculum (unsterilized and sterilized soil inoculum).

Because each inoculum might have had its own specific microbial community, all pots received 5 ml of a microbial wash. This wash was prepared by sieving 25 g of the soil mixture with 25 g of each inoculum in 5 L of demineralized water with a series of sieves. To ensure that only bacterial communities could penetrate the wash and to avoid fungal contamination, the finest of these sieves was 10 µm. We placed the pots in a climate controlled growth chamber with a 8:16 h dark:light cycle, a temperature of 16.2 ºC day:night and 60% humidity. We watered the pots every other day with tap water and randomly relocated the pots every week.

Harvest

We harvested the plants 12 weeks after inoculation and carefully shook the roots loose from the soil. We washed the plants with water, dried them with paper and separated roots from shoots. We further separated roots in two subsamples and weighted them. One of the subsamples was stored at 4 ºC for determination of AMF colonization (see below). The other subsample was dried at 70 ºC and reweighed to estimate the percentage of water loss. This value was used to predict the root dry mass of the subsample used for the determination of AMF colonization. Total root dry mass was then calculated by adding the root dry mass of the two subsamples. The shoots were also dried at 70 ºC and weighed. Total plant biomass was calculated by adding shoot and root dry mass.

AMF colonization

To determine the percentage of AMF colonization of roots, we cleared the fresh root subsamples with 10% KOH and stained them with 5% pen-ink vinegar both in a water bath (90 ºC) as described in (Vierheilig *et al.*, 1998). Stained roots were scored for the presence of AM fungi using the intersect method outlined in (McGonigle *et al.*, 1990). For each sample, 50-line intersections per root sample were scored for the presence of hyphae, vesicles or arbuscules. From these measurements the total percentage of root length colonized by AMF (which equals the amount of root length occupied by hyphae) was estimated.

Statistical analyses

According to the factorial design, we analyzed the main effects of plant species identity and AMF isolate identity and their interaction on plant biomass and percent root colonization. Specifically, we wanted to know if particular combinations of plant species and AMF isolates were resulting in higher or lower plant biomass and AMF root colonization then expected based on additive main effects. Such specific interactions would indicate potential specializations in plant–AMF associations. We further examined the relationship between AMF root colonization and plant biomass. All our analyses were carried out using the statistical software R (R version 2.13.0). Only roots that were inoculated with AMF were included in the corresponding correlation analysis.

Results

Effects on plant biomass

Total plant biomass was affected by both the identity of plant species and of AMF isolates ($F_{6, 168}$= 295.28, $p < 0.001$ and Fig.1a; $F_{6, 168}$= 18.58, $p < 0.001$ and Fig.1b; F values for the main effects of plant species and AMF isolates, respectively, after fitting contrast "control vs. AMF"). In the non-mycorrhizal controls, *P. lanceolata* and *L. corniculatus* had

the highest and *A. vineale* the lowest plant biomass. The interaction between plant species and AMF isolates, which addressed our main question of specificity in the plant–AMF association, had a strong effect on plant biomass ($F_{36, 168}$= 7.98, p < 0.001, after fitting contrast "control vs. AMF x plant species").

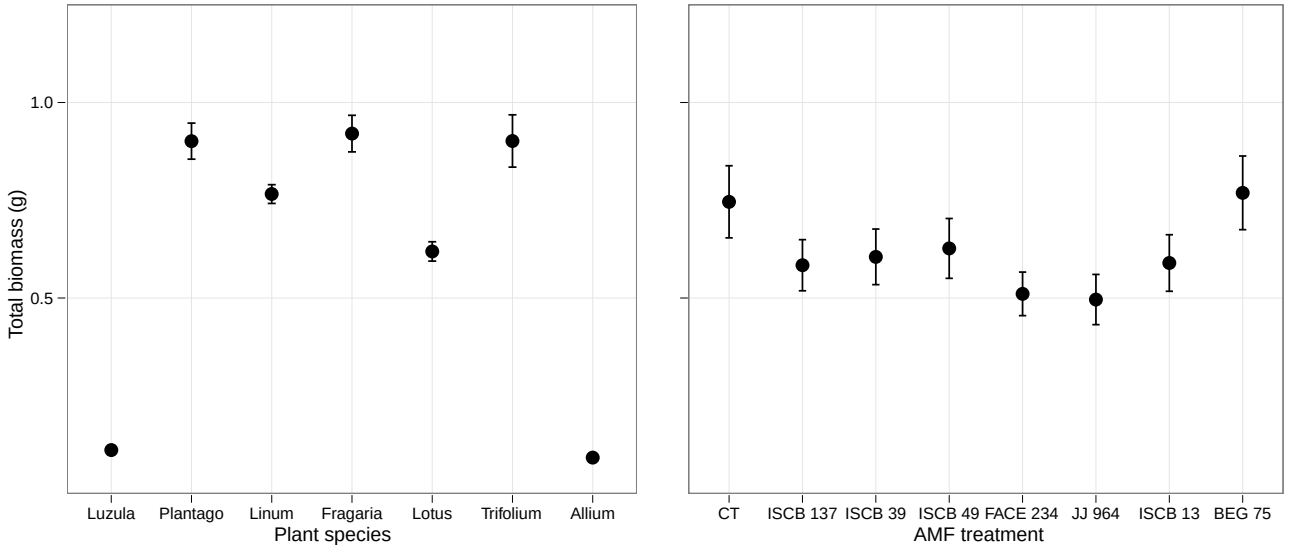


Figure 1.a) Total plant biomass (mean across control and AMF isolates ± 1 SEM, n=32) for every plant species. **b)** Total plant biomass (mean across plant species ± 1 SEM, n=28) for every AMF treatment (CT = control without AMF).

To analyze this interaction further, we plotted it as deviation matrix in Fig. 2. The deviations were calculated as the differences between the estimates of the model without (additive main effects) and the model with interaction. Large positive and negative deviations were most evident for the plant species *T. pratense.* Using an additional contrast for this species explained a large part of the interaction ($F_{6, 168}$= 42.53, p < 0.0001 for contrast "*T. pratense* vs. other plant species x AMF isolates" and $F_{30, 168}$= 2.85, p < 0.0001 for "other plant species x AMF isolates"). The plant species *P. lanceolata* and to a lesser extent *F. vesca* also responded differently to the seven AMF isolates than predicted from additive main effects, but for the other four plant species no such specialization in the association with particular AMF

isolates could be observed. In the following we will focus on the comparison between *P. lanceolata* and *T. pratense*.

These two plant species showed a highly complementary pattern of positive and negative associations with particular fungal isolates, with the sign of the interaction being the opposite for almost every AMF isolate (Fig. 2). For *T. pratense,* the largest positive interaction was found in the treatment inoculated with the isolate BEG 75 (*R. irregularis*), while for *P. lanceolata* it was with the isolate ISCB 39 (*C. etunicatum*). On the other hand, the largest negative interaction for *T. pratense* was found in the treatment inoculated with AMF isolate ISCB 49 (*C. lamellosum*) while for *P. lanceolata* it was with the isolate JJ 964 (*F. mosseae*).

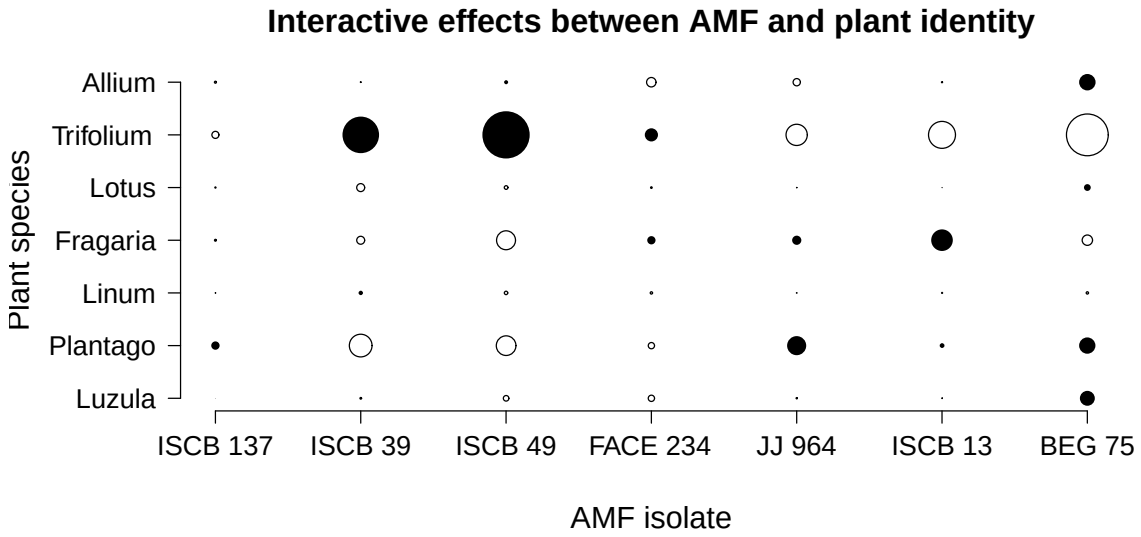


Figure 2. Interactive effects for every plant species–AMF treatment combination for plant biomass. The size of the circles represents the magnitude of the deviation from predictions based on additive main effects. Open circles represent positive deviations and filled circles represent negative deviations.

AMF colonization

We did not find any root colonized with AMF from treatments inoculated with the non-mycorrhizal control treatment. Furthermore, the non-mycorrhizal species *L. campestris* did not show AMF infection signs in any inoculation treatment. Focusing again on *P.*

lanceolata and *T. pratense*, no signs of colonization were found with ISCB 49 for both plant species and with ISCB 39 for *P. lanceolata*; in contrast high percentages of colonization were found for the two isolates of *R. irregularis* (ISCB 137 and BEG 75) on both plant species (Fig. 3). In addition to these colonization differences among the seven AMF isolates on the two plant species ($F_{6, 42}$= 313.72, p< 0.001), infection percentages were on average lower in *P. lanceolata* than in *T. pratense* ($F_{1, 42}$= 82.76, p< 0.001). Finally, there was a significant interaction "plant species x AMF isolates", indicating a differential response of *P. lanceolata* and *T. pratense* to the spectrum of AMF isolates ($F_{6, 42}$= 136.94, p< 0.001; Fig. 3).

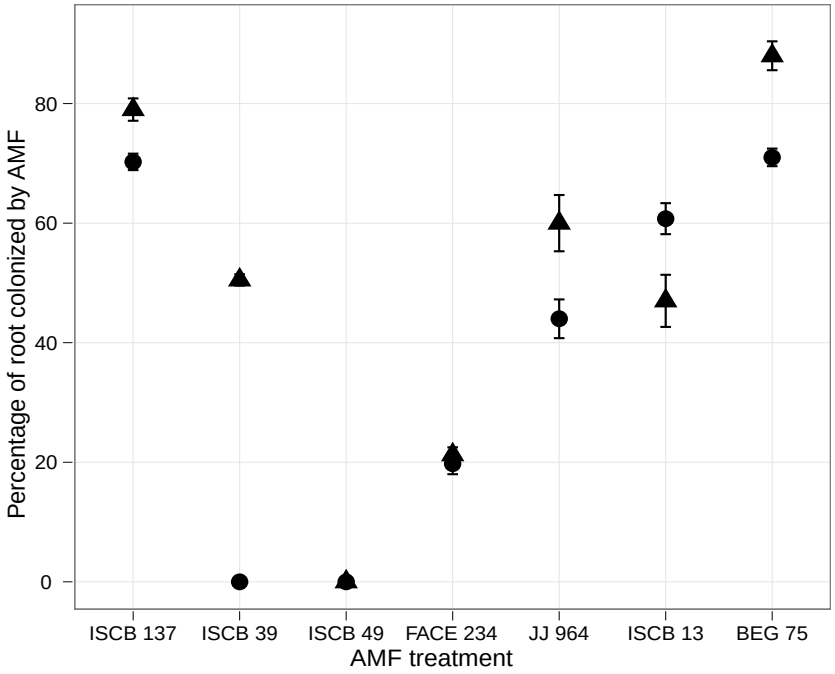


Figure 3. Percentage of root colonization (mean ± 1 SEM, n=4) by the seven AMF isolates on. *P. lanceolata*. (circles) and *T. pratense* (triangles).

Again, this differential response was reflected in a complementary pattern of positive and negative associations of *P. lanceolata* and *T. pratense* with the different AMF isolates (Fig.4a).

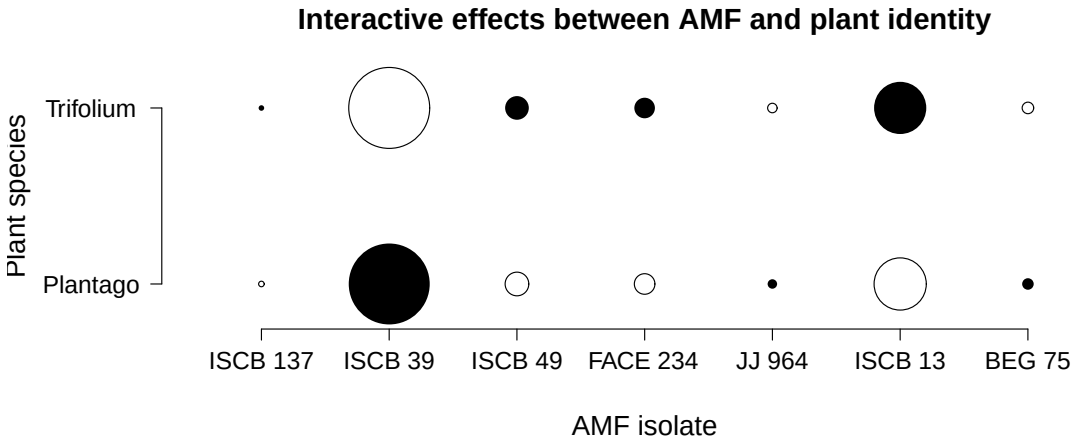


Figure 4. Interactive effects for *P. lanceolata* and *T. pratense* with the seven AMF isolates for percentage of root colonization. The size of the circles represents the magnitude of the deviation from predictions based on additive main effects. Open circles represent positive deviations and filled circles represent negative deviations.

Interestingly, the complementary patterns of deviations between the two plant species for plant biomass (Fig. 2) and percentage root colonization (Fig. 4) do not coincide well. Thus, absence vs. presence of colonization with AMF isolate ISCB 39 caused positive vs. negative biomass deviations in *P. lanceolota* vs. *T. pratense* suggesting a plant–AMF match with negative consequences for the plant *T. pratense*. Another match with similarly negative consequences, but with the plant species exchanged, was observed for AMF isolate ISCB 13: a higher than expected root colonization in *P. lanceolota* was correlated with a (slightly) lower than expected plant biomass (and a lower than expected root colonization in *T. pratense* was correlated with a higher than expected plant biomass). Ignoring these specific interactions with particular AMF isolates, the two plant species also showed different overall correlations of plant biomass with percentage root colonization by AMF (Fig. 5). Whereas the correlation was positive in *T. pratense* (R^2=0.51, p=<0.001) it was negative for *P. lanceolata* (R^2=0.34, p=0.001). A logistic regression furthermore showed that the simple infection by AMF reduced plant biomass in *P. lanceolota* and increased it in *T. pratense* ($F_{1, 52}$=, p=< 0.001).

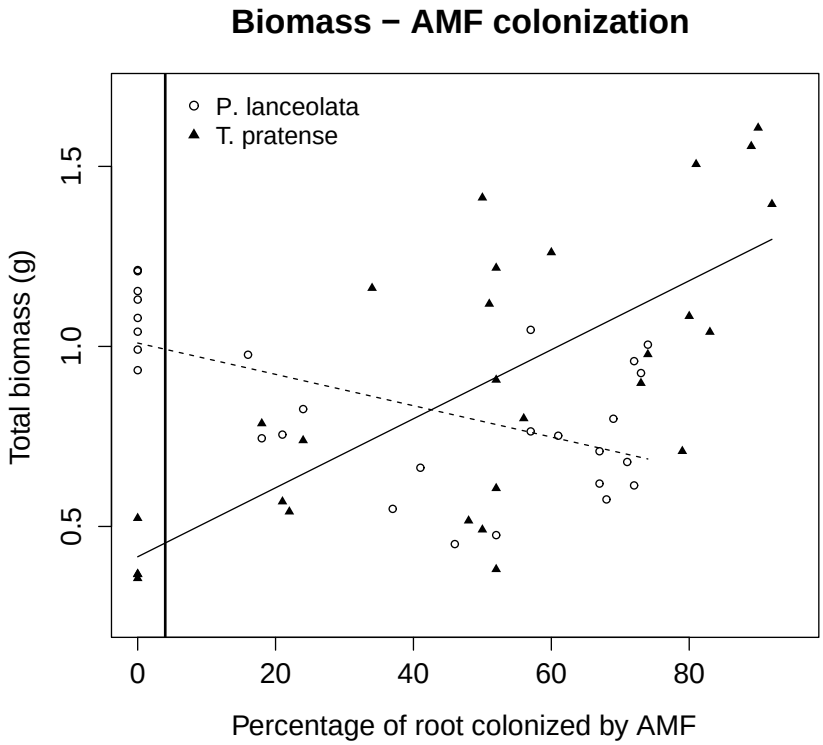


Figure 5. Correlation between total biomass and percentage of root colonized by AMF for *P. lanceolata* and *T. pratense***.**

Discussion

Our results emphasize that plant–AMF associations are more than a simple mutualistic symbiosis with a uniform response over all AMF and plants species. We found large differences in plant responses (i.e. total plant biomass) and AMF colonization rates among 56 different plant–AMF combinations, in line with previous studies showing differential plant response to mycorrhizal colonization (Van der Heijden *et al.*, 1998; Klironomos, 2003). Of the seven plant species investigated, only *A. vineale* was obligatory dependent on AMF for its growth, while in general the effect of AMF on plant biomass was neutral or negative although with large variation among plant–AMF combinations.

The roots of plant species with a high mycorrhizal dependency are often thick and unbranched, with few root hairs, and not well adapted to acquire nutrients (Baylis, 1975;

Hetrick *et al.*, 1992; Newsham *et al.*, 1995), like those of *A. vineale*. Also, legumes are known to by highly responsive to mycorrhizal inoculation (Wilson & Hartnett, 1998; Scheublin *et al.*, 2004). This high mycorrhizal dependency of legumes probably reflects the relatively higher phosphorous (P) demand caused by the nitrogen-fixation process (Azcón-Aguilar, 1992). However, of the two legumes species used in our experiment (*T. pratense* and *L. corniculatus*) only *T. pratense* showed positive responses to AMF colonization and not even for all the AMF isolates.

We hypothesize that the generally low beneficial effect caused by AMF in the growth of all seven plant species in our experiment might be due to the very low amount of nutrients in the growth medium. We used a nutrient-poor sandy soil and there was no addition of nutrients during the experiment. When nutrients are limited for both the plants and also for the AMF, the symbiosis might not be beneficial and result in competition between partners for the scarce nutrients. Due to the larger absorptive area of AMF hyphae compared to roots, AMF may in fact outcompete the plants in the process of nutrient acquisition (Johnson *et al.*, 1997; Treseder & Allen, 2002)We furthermore showed that the interactions among AMF and plant identity varied not only in their magnitude but also in their direction. Because AMF isolates can vary significantly in their growing strategies (Hart & Reader, 2002) they can also influence the plant growth and development (Sanders & Fitter, 1992; Streitwolf-Engel *et al.*, 1997; van der Heijden *et al.*, 1998; Klironomos, 2003). However, so far it has not been possible to classify AMF isolates as mutualists or parasites, since their influence on plant growth is highly dependent on the plant genotype with which they are associated (Klironomos, 2003) or the environmental conditions (Johnson *et al.*, 1997; Kiers & Van Der Heijden, 2006).

In our study, *P. lanceolata* and *T.pratense* explained most of the interactive effects between plant and AMF identity but, interestingly, following opposite directions for most of

the AMF isolates. It has been previously hypothesized that AMF might have differential feedback effects on plants (Bever, 1999). Hence, a feedback response would occur when an AMF isolate affects the growth of a plants species, which, in turn, has a positive (or negative) effect on the performance of the AMF. For *T. pratense,* the largest positive interaction was found in the treatment inoculated with the isolate BEG 75 (*R. irregularis*) while for *P. lanceolata* it was with the isolate ISCB 39, which, however, did not colonize the roots of *P. lanceolata*. The existence of different feedbacks for plant–AMF associations were confirmed by the significantly higher colonization rates for *T. pratense* than for *P. lanceolata* and by the different sign in the correlations between AMF colonization rates and total biomass. While higher colonization rates were in general positively correlated with greater plant biomass for *T. pratense,* this relationship was negative for *P. lanceolata.* However, the complementary patterns of deviations between the two plant species for plant biomass and percentage root colonization did not apply for the isolates ISCB 39 (absence vs. presence of colonization caused positive vs. negative biomass deviations in *P. lanceolota* vs. *T. pratense)* and ISCB 13 (a higher than expected root colonization in *P. lanceolota* was correlated with a (slightly) lower than expected plant biomass and a lower than expected root colonization in *T. pratense* was correlated with a higher than expected plant biomass). These deviations suggest that specific matches between plants and AMF can sometimes have negative consequences for the plant.

Remarkable was also the negative interaction between *T. pratense* and the isolate ISCB 49. According to (Gange *et al.*, 1999), there cannot be any direct influence of the mycorrhizal partner on a plant which has not been colonized. In our study, *T. pratense* was not colonized by this AMF isolate but still showed a decrease in biomass in the presence of the ISCB 49. Such a response might be related to negative feedbacks with the soil from the inoculum possible containing pathogens (Zuppinger-Dingley *et al.*, 2011); but we did not carry out specific test for this.

Our results demonstrate that plant species vary in their interactions with AMF and that these interactions cause species-specific feedbacks, with both positive and negative signs for the interaction depending on the plant–AMF matches. Associations between plants and other organisms have been shown to present different levels of specialization. Mutualistic associations (e.g. plant–pollinator, plant–frugivore and plant–ant systems) are often characterized by asymmetric specialization where specialist species on one side tend to interact with the most generalist species on the other (Bascompte *et al.*, 2006). These positive interactions can be enhanced through co-evolutionary convergence and complementarity of traits (J. N. Thompson, 2005; Bascompte *et al.*, 2006; Guimarães *et al.*, 2006; Thebault & Fontaine, 2008). On the other hand, antagonistic interactions (e.g. plant–consumers, plant–pathogens) are presumed to favor symmetric specialization and compartmentalization through the persistent co-evolution of defenses and counter defenses that cause greater reciprocal adaptation (Thompson, 2005). For the plant–AMF association, modulation of plant defenses is required for the establishment of the symbiosis, and there is evidence for the accumulation of defensive plant compounds related to mycorrhizal colonization, although to a much lower extent than in plant–pathogen interactions (Pozo & Azcón-Aguilar, 2007).

Because, as shown in our results, in the plant–AMF symbiosis both negative and positive specific interactions are possible, several ecological and co-evolutionary mechanisms are probably involved in the mechanisms controlling the association and the specialization process. Accordingly, interspecific interactions between plants and AMFs and their feedbacks may influence the relative performances of multiple plant species and AMF in a community (Bever *et al.*, 1997; Klironomos, 2002)and play an important role in mediating plant species coexistence and maintaining local biodiversity (Callaway & Maron, 2006). Consequently, understanding how these interactions and the following feedbacks are affected by changes in the environment (e.g. changes in nutrient availability) or when multiple feedbacks co-occur at the individual level (e.g. AMF colonization by more than one AMF isolate for the same plant

individual) or community level (e.g. plant individuals linked by a common mycorrhizal network) is of great importance to determine the adaptive and ecological processes at work in this bidirectional symbiosis and its consequences for ecosystem functioning.

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Supplement

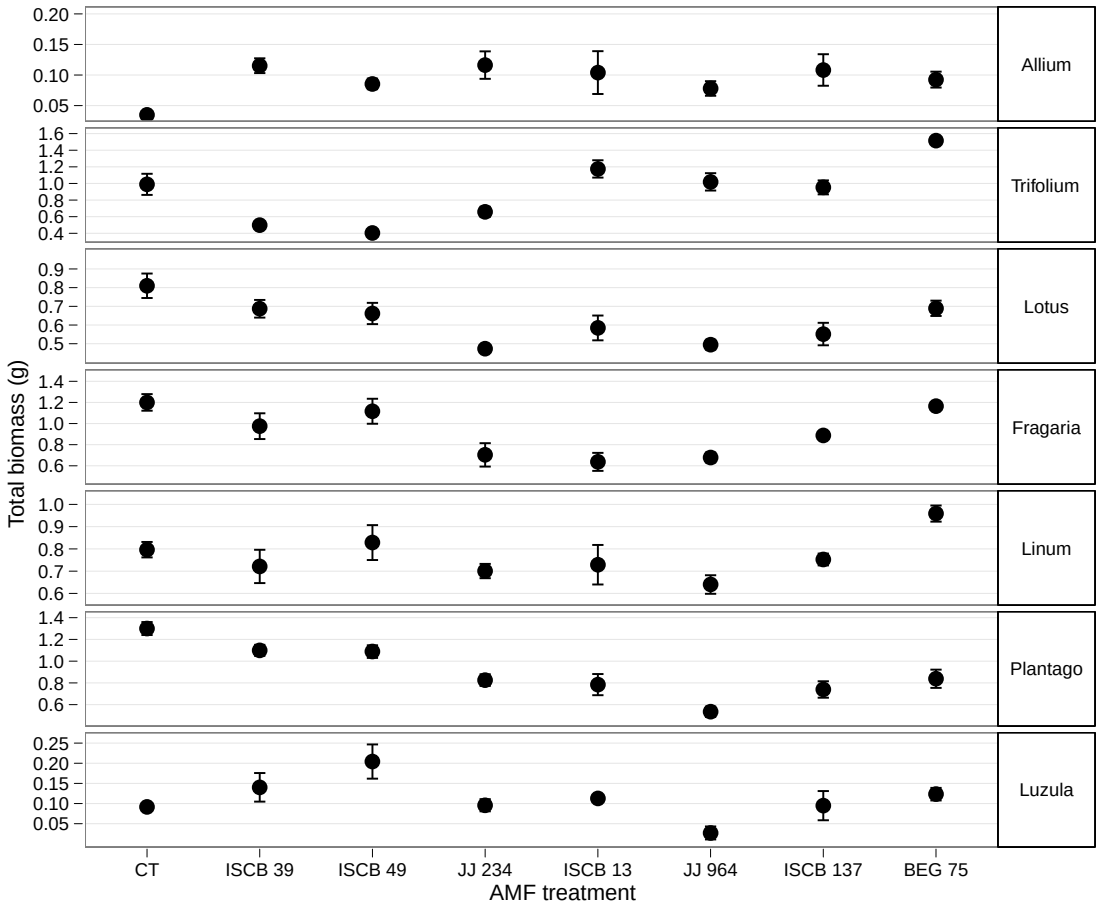
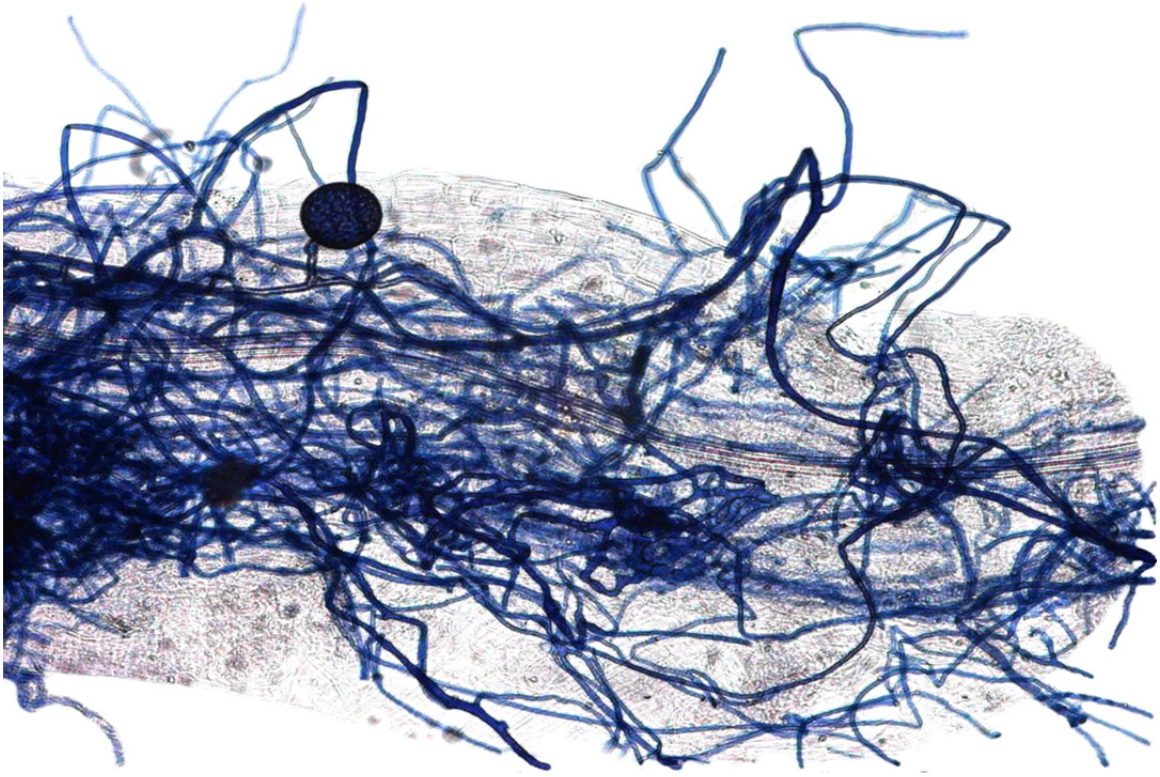


Figure S1. Total biomass (mean ± 1 SEM, n=4) for every plant species – AMF treatment combination.

Chapter Two

Effects of mycorrhizal combination treatments on plants with split-root systems

Alicia Argüello, Marcel van der Heijden, Andres Wiemken and Bernhard Schmid



Abstract

The effects of arbuscular mycorrhizal fungi (AMF) on the productivity of their associated host plants largely depend on AMF identity. Cooperative associations between plants and AMF are furthermore thought to be favored by highly spatially structured systems; however, this belief has recently been challenged by new observations. In this study, we analysed the influence of spatial structure in AMF presence on the plant *Trifolium pratense* and its ability to select between two different AMF species. The spatial structure in AMF presence was achieved by combining the two AMF species or non-mycorrhizal control treatments in split-root systems of single test plants. We found that the two AMF species had similar and positive effects when AMF were present in both halves of the split-root systems, including the mixture of the two AMF. However, while an increased presence (no AMF < AMF in one half < AMF in both halves or the split-root system) of the supposedly more beneficial AMF species *(Diversispora celata)* enhanced plant biomass and shoot-root ratio, the full positive effect of the supposedly less beneficial AMF species *(Funneliformis mosseae)* was already reached when only one half of the split-root system was inoculated. In treatments where AMF were only present in one compartment of a split-root system, the plant allocated more root biomass to this side, indicating a general preference towards AMF. In split-root systems with homogeneous AMF combinations plants infected with *D. celata* had slightly less root biomass than plants infected with *F. mosseae.* However, in split-root systems with heterogeneous AMF combinations this was reversed and the compartment with *D. celata* had more root biomass than the compartment *F. mosseae.* This suggests that plants of *T. pratense* can select between AMF partners present in different parts of the root system, even though they do not discriminate when only one of them is present.

Key words: AMF-selective plants, *Diversispora celata*, *Funneliformis mosseae*, preferential root allocation, *Trifolium pratense*.

Introduction

Arbuscular mycorrhizal fungi (AMF) are associated with the vast majority of plant species. The beneficial effects of this association for a plant species depend on the identity of the AMF species (Bever, 2002; van der Heijden *et al.*, 2003). Therefore, changes in the composition of AMF species in an ecosystem may trigger a cascade of effects influencing, for example, nutrient availability, plant community biomass and the relative abundance of plant species (Bever, 2002; Girlanda *et al.*, 2006; Wagg *et al.*, 2011).

In plant–AMF associations, host plants are typically infected by multiple AMF species (Vandenkoornhuyse *et al.*, 2002; Scheublin *et al.*, 2004). Under these conditions enforcement mechanisms against poor-quality partners may stabilize cooperation (Kiers *et al.*, 2007). However, there is still little empirical evidence from work on plant–AMF associations (Bever *et al.*, 2009; Kiers *et al.*, 2011; Verbruggen *et al.*, 2012) which supports this idea of partner choice as an element for the maintenance of mutualistic associations (Denison *et al.*, 2003; Kiers & Van Der Heijden, 2006). Furthermore, recent work has shown that spatial structuring can select against cooperation: when spatial structure was reduced by mixing soil, more beneficial AMF species could be discriminated more easily from less beneficial ones by the plant root system (Verbruggen *et al.*, 2012). This contrasts with the more traditional speculation that high spatial structuring may be critical for stabilizing cooperation in plant–AMF associations (Chanway *et al.*, 1991; Wilkinson, 1998; Bever *et al.*, 2009; Hodge & Fitter, 2010).

Species-specific effects in plant–AMF associations have been demonstrated in a number of studies from temperate grasslands (Bever *et al.*, 1996; van der Heijden *et al.*, 1998, Chapter "Interactive effects"). On the side of the AMF partner, AMF species composition and AMF species richness can play a major role in the maintenance of plant community diversity and in plant community productivity in experimental settings (van der Heijden *et al.*, 1998b; Bever *et al.*, 2001; van der Heijden & Sanders, 2002). In the field, differences in AMF communities have been found between plant species, ecosystems, locations, seasons (Bever *et al.*, 2001; Husband *et al.*, 2002; Opik *et al.*, 2006) and even between different parts of the root system of single plant individuals (Scheublin *et al.*, 2004). However, it is difficult to link these studies about the composition of AMF communities in the field with experimental studies about the functional significance of these AMF communities for plant growth and subsequent plant community diversity and productivity.

Recently, Wagg *et al.* (2011) demonstrated that increased diversity of AMF fungi can promote plant growth and coexistence, and that selection and complementarity effects among AMF species can raise plant community productivity above the average obtained with single AMF species. Hence, they investigated the relative contribution to aboveground plant productivity of each AMF species in monocultures and in mixture. One of the AMF isolates, FACE 234 (*Diversispora celata*), was particularly beneficial for plant growth in *Trifolium pratense*, while another AMF isolate, JJ 964 (*Funneliformis mosseae* previously name *Glomus mosseae* (Krüger *et al.*, 2012)), was least beneficial and even had a negative effect when combined with FACE 234. These observations were made in systems where the different AMF species were well mixed. In the present study we tested if similar

effects were also possible when different AMF species were separately associated with different parts of a plant root system, i.e. with high spatial structuring.

In our study, we used the same plant species (*Trifolium pratense*) and two of the same AMF species (isolates FACE 234 and JJ 964) as Wagg *et al.* (2011) had used. A non-mycorrhizal treatment allowed us to test whether AMF effects depended on AMF presence, identity and combination in split root systems where a single plant had roots in two separate compartments. We asked whether spatial separation would change AMF effects and whether plants accordingly could select between different AMF species by allocating biomass differentially to the two compartments.

Material and Methods

Experimental setup

We built split-root systems in which roots of individual plants could be divided between two compartments (Fig. 1). Each compartment consisted of two 400-cm^3 square plastic pots stacked inside each other to provide stability and separated by a plastic film to prevent root propagation outside the compartment. To form the split-root system we taped two of these compartments together side by side. To support the stem, the plant was grown with a 3-cm long PVC tube which rested on the soil surface. This design allowed us to separately control the conditions in each compartment of the split-root system.

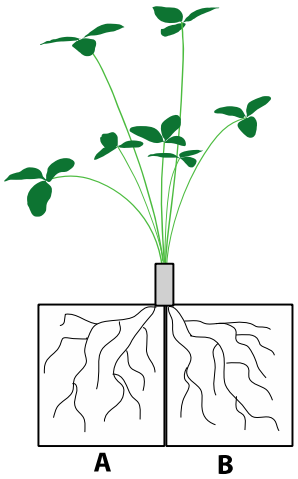


Figure 1. Schematic of the split-root systems used in the experiment with A and B representing the two compartments.

Each of the two compartments received one of three different AMF treatments: *Diversispora celata* inoculum (isolate FACE 234, abbreviated FA; (Gamper *et al.*, 2009)), *Funneliformis mosseae* inoculum (isolate JJ 964, abbreviated JJ; (Krüger *et al.*, 2012)) and a non-mycorrhizal control inoculum (CT). The isolate FACE 234 was previously reported as particularly beneficial for plant growth in *Trifolium pratense*, while JJ 964 was proved as least beneficial and even had a negative effect when combined with FACE 234 (Wagg *et al.*, 2011). These three AMF treatments could be applied in six different ways to the paired split-root systems, yielding six AMF combination treatments at the level of an entire split-root system. In three of these combinations (CT-CT, FA-FA and JJ-JJ), both compartments had the same AMF treatment. In the remaining three combinations (CT-FA, CT-JJ and FA-JJ), the two compartments received different AMF treatments. Each of the six AMF combination treatments was replicated eight times for the test plant species *Trifolium pratense*, giving 48 split-root systems in total.

Growth medium

We used a 1:9 volumes of a field-soil:quartz-sand mixture as growth medium. The field soil was collected from a natural grass-clover field at the Agroscope Reckenholz research station in Zürich, Switzerland (47° 25'N, 8°31'E), and sieved through a 5 mm mesh before mixing. The mixture had a pH of 6.7 and was sterilized with gamma radiation at ca. 50 kGy (range 25–80 kGy LEONI, Aargau, Switzerland).

Biological material

Seeds of *T. pratense* were obtained from agricultural plots located at Agroscope Reckenholz research station, Zürich, Switzerland. They were pretreated in 5% chloride for 10 minutes and thoroughly rinsed four times with demineralized water. Seeds were allowed to germinate in sterile sand in May 2009. These seedlings produced one main root and hence, to stimulate the outgrowth of several lateral roots which could be bent into separate directions to form a split-root system afterwards, the main root was cut around 2 cm below the shoot. The seedlings were first transplanted individually to small pots to avoid competition and after 4 weeks one plant each was transplanted to a single pot-pair described above to establish the split-root systems. These were placed in a glasshouse compartment in mid June 2009 with a 8:16 h dark:light cycle, a temperature of 16:21 ºC day:night and 60% humidity.

For the AMF treatment, a predefined mass of soil inoculum was mixed with the soil such that approximately 100 spores were present per compartment with AMF. One gram of inoculum contained 15 spores in the case of FACE 234 and 5 spores in the case of JJ 964. Differences in inoculum volumes were corrected by adding a sterilized (autoclaved for 60 min at 121 °C) mixture of the two AMF isolates to avoid

confounding effects due to different nutrient levels. This sterilized mixture was also used as inoculum for the non-mycorrhizal control treatment.

Because the different inocula might have had different microbial communities, all pots received additionally 5 ml of a standardized microbial wash from both types. This microbial wash was prepared by sieving 25 g of the non-sterilized growth medium with 25 g of both FACE 234 and JJ 964 inoculum in 5 L of distilled water with a series of sieves. To ensure that only bacterial communities could penetrate the wash and to avoid fungal contamination, the finest of these sieves was 10 µm. Many root nodules were observed on the host plant *T. pratense* indicating that this microbial wash contained active microorganisms including nodule-inducing rhizobial species.

We inoculated the soils in the two compartments when planting the split-root plant individuals into them. We watered the pots every other day with tap water and added 5 ml of a nutrient solution every week until a total of 50 ml. This solution was a modified Hoagland solution (Hoagland & Arnon, 1950) with half of the normal P concentration (6 mM KNO_3; 4mM $Ca(NO_3)_2$; 0,5 mM NH_4NO_3; 1mM $NH_4H_2PO_4$; 1mM $MgSO_4$; 50 µM KCl; 25 µM H_3BO_3; 2 µM $MnSO_4$; 2 µM $ZnSO_4$; 0,5 µM $CuSO_4$; 0,5µM $(NH_4)6Mo7O_{24}$; 20 µM Fe(Na)EDTA). This solution was applied just before the plants were watered, to make sure that the nutrients mixed well with the soil. We randomly relocated the split-root systems every week on the glasshouse benches.

Harvest

We harvested the plants after 12 weeks of growth in the split-root systems and carefully shook the roots loose from the soil. We washed the plants with water, dried them with paper and separated roots from shoots. We further separated roots

according to compartments and within compartments into two subsamples each and weighed them fresh. One of the subsamples per compartment was stored at 4 ºC for determination of AMF colonization (see below). The other subsample was dried at 70 ºC and reweighed to estimate the percentage of water loss. This value was used to predict the root dry mass of the subsample used for AMF colonization determination. Root dry mass per compartment was then calculated adding the root dry mass of both subsamples. The shoots were equally dried at 70 ºC and weighed. Total plant biomass was calculated adding the weight of the root dry mass from both compartments and of the shoot dry mass.

AMF colonization

For the determination of AMF colonization, fresh root subsamples were cleared with 10% KOH and stained with 5% pen-ink vinegar in a hot water bath (90 ºC) as described (Vierheilig *et al.*, 1998). Stained roots were scored for the amount of colonization by AMF using the intersect method outlined in McGonigle *et al.* (1990). For each sample, 50 line intersections per root sample were scored for the presence of hyphae, vesicles, and arbuscules. From these measurements, the total percentage of root length colonized by AMF (which equals the amount of root length occupied by hyphae) was estimated.

Statistical analyses

We investigated the effects of AMF combination treatments on the total plant biomass and shoot-root ratio of *T. pratense*. Using linear models, we compared the root biomass and percentage of root colonization for each compartment based on the treatment applied in one compartment considered as the target and the treatment in the other compartment considered as the neighbor within a split-root system. All of our

analyses were carried out using the statistical software R (R version 2.13.0). Only roots with AMF colonization > 0 were included in the analysis of effects of percentage colonization on plant biomass.

Results

Effects of AMF combination treatments on total plant biomass

Trifolium pratense responded differently to the six AMF combination treatments (Fig. 1; $F_{5, 42} = 2.85$, $p = 0.027$). We found an overall positive effect of the presence of AMF on total plant biomass ($t = –2.12$, $p = 0.040$ for the contrast between CT-CT and the remaining five treatments, all with presence of AMF). FACE 234 (*D. celata*) yielded higher biomass than the control treatment (CT-CT) when it was present in both compartments ($t = 2.24$, $p = 0.030$ for the contrast between CT-CT and FA-FA). For JJ 964 (*F. mosseae*), we found higher biomass both when it was present just in one compartment ($t = 2.68$, $p = 0.010$ for the contrast between CT-CT and CT-JJ) or in both compartments ($t = 2.249$, $p = 0.029$ for the contrast between CT-CT and JJ-JJ). There was no significant variation in biomass among treatments where AMF were present in both compartments ($F_{2, 21} = 0.83$, $p = 0.451$ for three-way comparison among FA-FA, FA-JJ and JJ-JJ).

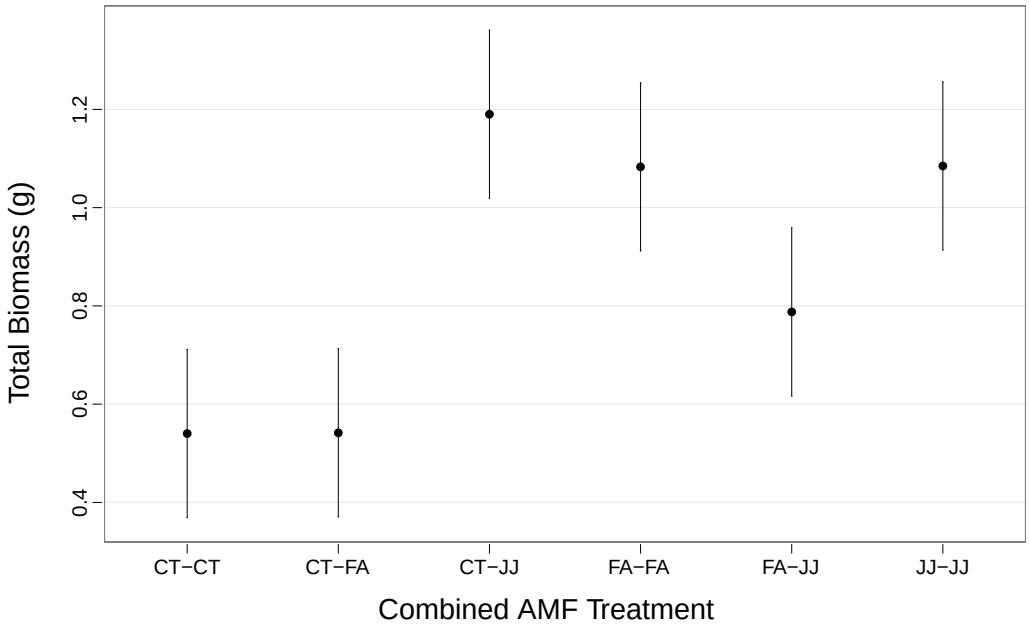


Figure 1. Effect of AMF combination treatments (CT = control without AMF, FA = *Diversispora celata*, JJ = *Funneliformis mosseae*) on total plant biomass of *Trifolium pratense*. Data shown (mean ± s.e. [standard error of mean]) are based on the fitted linear model.

Effects of AMF combination treatments on shoot-root ratios

The ratio between shoot and (total) root biomass varied significantly between the six AMF combination treatments, with lowest values when AMF were absent in both compartments and highest values when they were present in both compartments (Fig. 2; $F_{5, 42} = 2.59$, $p = 0.039$; $t = –2.85$, $p = 0.007$ for the contrast between CT-CT and the remaining five treatments, all with presence of AMF). The shoot-root ratio for those treatments where the isolate FACE 234 (*D. celata*) was present just in one of the two compartments was marginally lower than for the remaining four treatments with presence of AMF ($t = –1.76$, $p = 0.086$ for corresponding contrast).

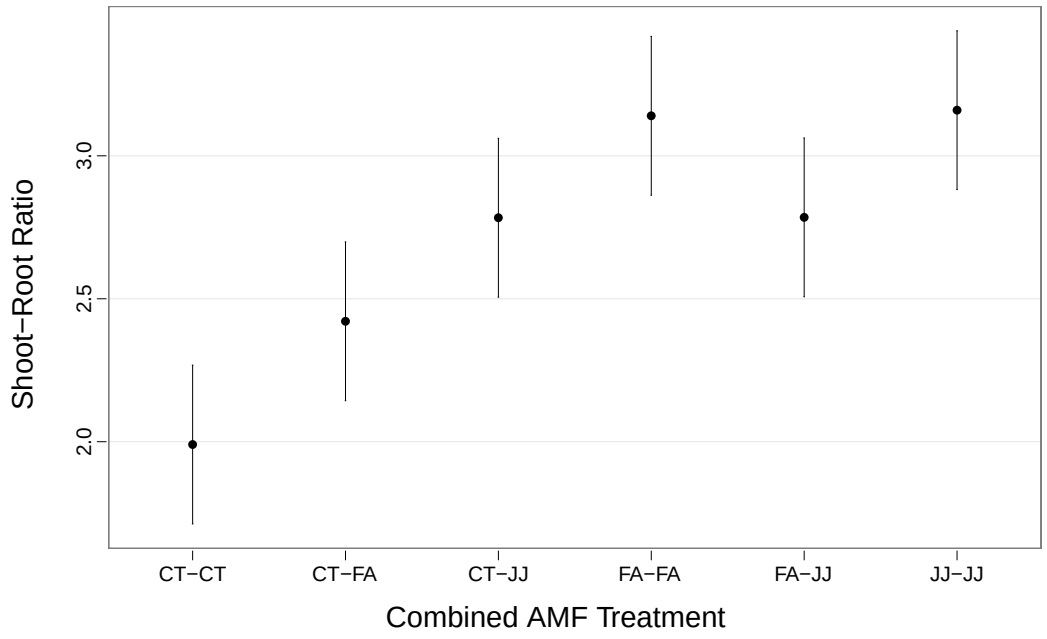


Figure 2. Effect of AMF combination treatments (CT = control without AMF, FA = *Diversispora celata*, JJ = *Funneliformis mosseae*) on shoot-root ratios of *Trifolium pratense*. Data shown (mean ± s.e.) are based on the fitted linear model.

Effects of AMF treatments on root biomass in single compartments

We found marginally larger absolute differences in root biomass between the compartments of heterogeneous AMF combination treatments than between the compartments of homogeneous AMF combination treatments ($F_{1, 33} = 3.62$, $p = 0.08$). The three heterogeneous AMF combination treatments also varied among themselves in the absolute differences in root biomass between compartments ($F_{2, 33} = 3.62$, $p = 0.038$). Furthermore we analysed root biomass in each single compartment of the split-root systems as a function of AMF treatments. Both the treatment applied in the target compartment and the treatment applied in the neighbour compartment of the split root system independently affected root biomass in the target compartment ($F_{2, 80} = 4.04$, $p = 0.021$ for AMF treatment in target compartment and $F_{2, 80} = 2.64$, $p =$

0.077 for AMF treatment in neighbour compartment). Root biomass was lower in the target compartment when it contained no AMF ($F_{1, 80} = 7.75$, $p = 0.007$, Fig.3), and also lower when the neighbour compartment contained FACE 234 ($F_{1, 80} = 4.86$, $p = 0.030$, Fig.3). This means that root biomasses differed between target and neighbour compartments in two of the heterogeneous AMF combination treatments (CT-FA, FA-JJ) in a compensatory way, with the heavier side being heavier and the lighter side being lighter than in the corresponding homogenous treatments (Fig. 3).

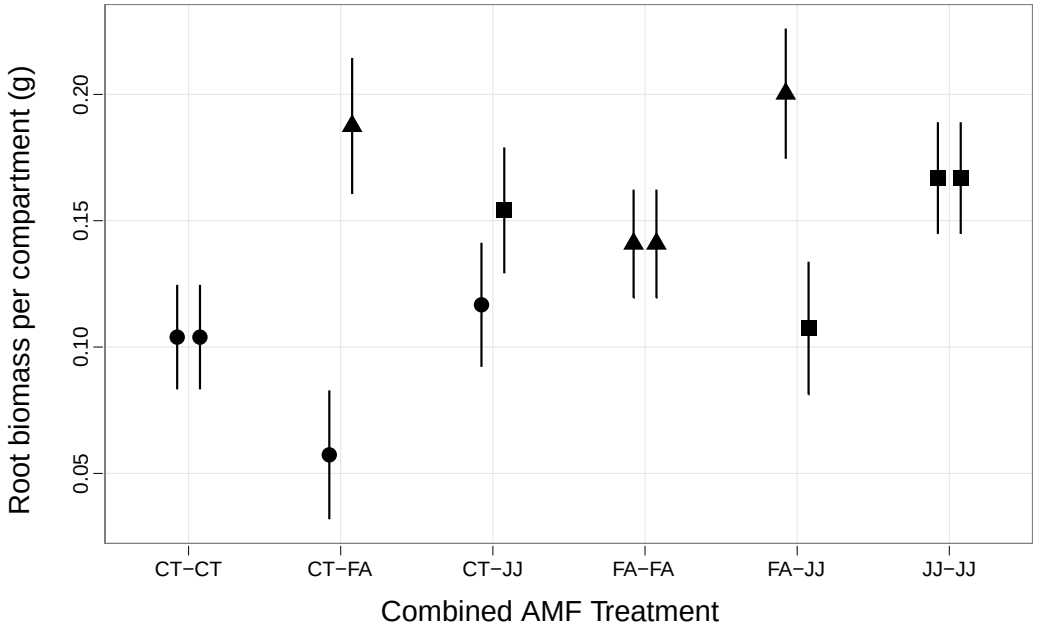


Figure 3. Effect of AMF treatments (circles = CT = control without AMF, triangles = FA = *Diversispora celata*, squares = JJ = *Funneliformis mosseae*) on plant root biomass for each compartment. Data shown (mean ± s.e.) are based on the fitted linear model.

Effects of AMF treatments on AMF colonization levels

No signs of colonization were found in the roots from compartments where the non-mycorrhizal control (CT) was applied, indicating that no external AMF contamination occurred (Fig. 4). The percentage of AMF colonization was significantly lower for the isolate FACE 234 (*D. celata*; 11.8% ± 1.3% [mean ± s.e.])

than for the isolate JJ 964 (*F. mosseae*; 62.7% ± 1.8%; $F_{1, 51} = 1186$, $p < 0.001$ for difference), but no correlations between total biomass and infection levels were found for any of the two AMF isolates. The percentage of root colonized in one compartment was also influenced by the treatment applied in the other compartment. In particular, a lower colonization rate was detected in the target compartment when JJ 964 (*F. mosseae*) was applied to the neighbour compartment ($F_{2, 74} = 8.01$, $p < 0.001$ for AMF treatment in neighbour compartment; Fig. 4).

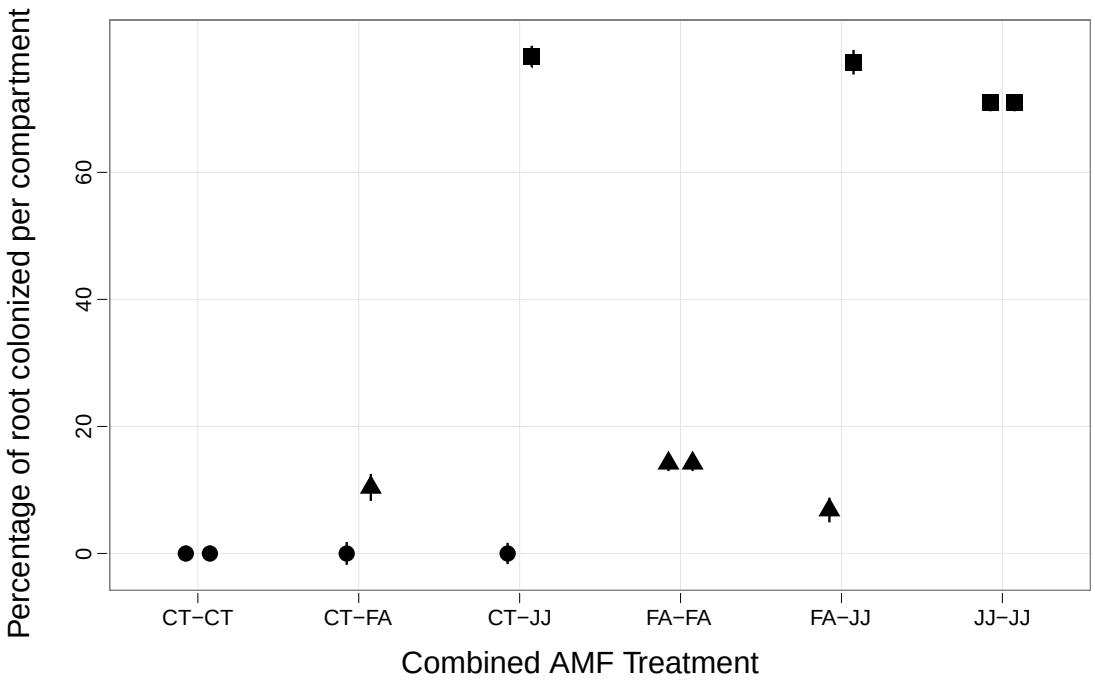


Figure 4. Effect of AMF treatments (circles = CT = control without AMF, triangles = FA = *Diversispora celata*, squares = JJ = *Funneliformis mosseae*) on AMF colonization for each compartment. Data shown (mean ± s.e.) are based on the fitted linear model.

Discussion

It is generally accepted that AMF effects on plant productivity can depend on AMF identity and that spatially structured systems can favor cooperation in mutualistic interactions (Bever & Simms, 2000; Hoeksema & Kummel, 2003;

Gardner *et al.*, 2007; Platt & Bever, 2009; Hodge & Fitter, 2010). However, in a recent study by Verbruggen *et al.* (2012), the second view was challenged and it was suggested that spatial structuring could select against cooperation. In our study, we found an overall positive effect of AMF on plant biomass, including an increased shoot-root ratio of whole plants. Nevertheless, in the highly spatially structured systems of our split-root design, the effects of the supposedly more beneficial mycorrhizal species *Diversispora celata* represented by isolate FACE 234 were not stronger than those of the supposedly less beneficial *Funneliformis mosseae* isolate JJ 964. This is in contrast to the findings by Wagg *et al.* (2011).

Although positive effects of increased AMF richness have been previously reported (Van der Heijden *et al.*, 1998; Maherali & Klironomos, 2007), we found that the benefit when we inoculated a plant with both AMF species (one in each compartment) was equal as when the plant was inoculated with each of the single AMF species separately. Thus, there were not significant differences among treatments in which both compartments contained AMF (FA-FA, FA-JJ and JJ-JJ). Our results are nevertheless in line with findings by Wagg *et al.* (2011) for the same AMF combinations. In their study, they found that dual inoculation with JJ 964 and FACE 234 resulted in either no difference (for a high-sand soil treatment) or lower biomass of *T. pratense* (for the low-sand soil treatment) than predicted from single inoculations with either of the two AMFs.

Increased fungal presence (in our case $0 < 1 < 2$ compartments with AMF) has been previously reported to enhance plant growth in split-root systems (Gustafson & Casper, 2005), either by increasing the total number of spores in the system or by inoculating both compartments. Interestingly, we found different effects for our two AMF isolates: while an increased presence of FACE 234 (*D. celata*) enhanced host

growth, the full positive effect of JJ 964 (*F. mosseae*) was already reached when only one compartment was inoculated. The lack of enhanced plant growth when JJ 964 was present in both compartments and the reduced infection percentages in target compartments when neighbour compartments contained JJ 964 might be explained by the presence of an auto-regulatory mechanism by the host plant. After a critical level of colonization, further root colonization by AMF in already mycorrhizal plants can be suppressed (Vierheilig *et al.*, 2000; Vierheilig, 2004). Also, the percentage of colonization is usually considered a measure of the performance of the symbiosis. Because for the isolate JJ 964 the percentage of colonization was much larger than for FACE 234, it could explain its greater effects compared to those of FACE 234 when colonizing just one of the two compartments.

Soil abiotic conditions may also influence AMF–plant relationships (Johnson *et al.*, 1997) and generally nutrient-poor sandy soils favor mutualistic responses. In their study, Wagg *et al.* (2011) found strong positive effects of AMF on plant productivity mainly driven by FACE 234 (*D. celata*), and especially in soil with a high sand content. Our soil had an even larger sand fraction (9:1) compared to the one used by Wagg *et al.* (2011) (3:1), but, interestingly, the positive effects of FACE 234 were the same or smaller (when present in only one compartment) than those of JJ 964 (*F. mosseae*). One possible explanation for these differences between studies may be due to the fact that, in our experiment, we added a modified Hoagland nutrient solution. Nutrient availability may affect AMF coexistence (Kennedy, 2010) and modify the effects of AMF on plant growth (Johnson *et al.*, 1997). Thus, although our solution contained only half of the normal phosphorous concentration, this might have altered the performance of the symbiotic association.

Similar to our findings in Chapter 4, the two halves of our split-plot root systems within single plants showed correlative responses to the presence of AMF. Using these split-root systems we found that plants modified their biomass allocation in opposite directions at the whole-plant (shoot-root ratios) and the root-system-half level (root biomass allocation to compartments). Hence, at the whole-plant level, plants produced more roots in absence of AMF (reduced shoot-root ratio), probably to enhance nutrient acquisition (Xu, 2010; Zhang *et al.*, 2011). However, at the root-system-half level, the presence of AMF, regardless of AMF identity, locally induced the production of roots in the target compartment. Furthermore, plants showed preferential allocation towards the isolate FACE 234, which itself had a negative effect on the root biomass of the target compartment when it was present in the neighbour compartment.

As mentioned before, root colonization is the proof of an active AMF symbiosis, and high percentages are usually associated with a higher level of resources exchange (Hodge & Fitter, 2010). Because the isolate FACE 234 involved lower colonization levels, it could result in lower carbon investment of the plant to the AMF, which could be used instead by the plant in building new roots, increasing the root biomass in these compartments. Because the effects of the treatments affected further than locally the biomass of the target compartment, we consider our results as an indication of a integrated plant response at the level of the entire root system. Our results, in line with previous findings (Chapter 4), show how belowground plasticity in biomass allocation to differently treated root halves is opposite to the individual plasticity in biomass allocation of whole plants with regard to both the nutrient and the AMF treatment.

Because belowground AMF diversity supports plant productivity and helps to maintain it under changing environmental conditions (Wagg *et al.*, 2011), it is important to be aware of how modifications of the spatial structure affect the different combinations of AMF. Whether spatial structure will stimulate or decrease mutualistic cooperation depends on the biology of the interaction (Verbruggen *et al.*, 2012).

In our study, under the high spatial structure of a split-root system, the two different AMF species had similarly positive effects when AMF were present in both compartments, including the mixture of the two AMF. Despite this, signs of preferential allocation (i.e. increased root biomass) were still observed for the AMF which a priori was considered to be more beneficial to the plant, FACE 234. Further empirical research is needed to (i) elucidate the mechanisms underlying the preferential host allocation, (ii) identify the actual benefits provided by the AMF (i.e. phosphorous or nitrogen transport) and (iii) investigate the effects of spatial structure for AMF combination treatments with other mycorrhizal and plant species.

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Chapter Three

Plants integrate spatially separated signals from AMF and nutrient availability and modify their biomass allocation accordingly

Alicia Argüello, Marcel van der Heijden, Andres Wiemken and Bernhard Schmid

Abstract

When associating with arbuscular mycorrhizal fungi (AMF), plants invest their own photosynthesized carbon into the fungal partner to obtain mineral nutrients from it. However, it is not known whether plants detect and potentially integrate specific signals from AMF or whether they only respond to the increased nutrient supply created by the AMF. To address this question we applied different combinations of AMF and nutrient treatments to the two halves of split-root systems of test plants and measured their responses at the level of the entire plant as biomass and nitrogen accumulation (referred to as performance) and as biomass allocation (referred to as plasticity) and at the level of the two halves of the root system as belowground biomass. We examined whether treatment effects were additive or interactive along a gradient from the least to the most beneficial conditions (linear increase from AMF in no to one to two compartments and from low-low to low-high to high-high nutrient combinations). Deviations from additivity were taken as indication of integrated responses at the level of the entire plant, with positive differences indicating positive effects of integration on plant performance. To account for the possible importance of plant species identity due to different mycorrhizal dependencies and nutrient requirements, we used two plant species from different functional groups, *Plantago lanceolata* and *Trifolium pratense*.

Spatially uniform AMF presence and nutrient availability positively affected plant performance. However, while in general *T. pratense* was mostly and positively affected by AMF presence, *P. lanceolata* was equally and additively affected by AMF presence and nutrient availability for biomass and negatively affected by AMF presence regarding nitrogen contents. In heterogeneous treatments, *P. lanceolata* showed positive integration for nutrient heterogeneity and, at low nutrient availability,

for AMF heterogeneity, whereas *T. pratense* showed no integration for nutrient heterogeneity and negative integration of AMF heterogeneity. Regarding plant plasticity, allocation percentages were biased towards roots in compartments with AMF or high nutrient levels. This can be seen as an integrated response because in homogeneous conditions entire plants showed the opposite plasticity response, namely reduced allocation of belowground biomass in presence of AMF or under high nutrient levels. We furthermore found correlated plant responses at the level of the two halves of the root system: AMF presence and nutrient increase in the neighbour compartment increased root biomass in the target compartment. The ability to respond precisely to spatial environmental variability was greater in *P. lanceolata* than in *T. pratense*. This may be related to greater plasticity in root foraging strategies of *P. lanceolata* and higher mycorrhizal (and rhizobial) dependency of *T. pratense.*

Key words: arbuscular mycorrhizal fungi (AMF), biomass allocation, correlative response, plant integration, root foraging, soil nutrients, spatial heterogeneity.

Introduction

As early as in the 4th century BC, Theophrastus described interactions among plants and their environment in his “On the causes of plants” (Ramalay, 1940). However, the environment to which a plant is exposed can be complex and heterogeneous. How different environmental factors in different combination and spatial arrangement affect plant fitness still remains a fundamental question in ecology. Many studies have been carried out to understand how plants respond to single and uniformly distributed environmental factors (Grime, 1979; Tilman, 1982; Taylor, 1990), yet the effects of multiple factors occurring simultaneously and

potentially in spatially heterogeneous arrangement are poorly understood (Reynolds *et al.*, 2003; van Kleunen & Fischer, 2005; Valladares *et al.*, 2007).

Plants are sessile organisms without central nervous system, but they still perceive environmental changes and respond to complex stress conditions (Schmid *et al.*, 1990; Schmid, 1992; Atkinson & Urwin, 2012). However, it is not clear to which extent plants can integrate signals from different factors or whether their response can be adjusted upon the temporal or the spatial variation in the occurrence or intensity of these factors (Shemesh *et al.*, 2010). The incorporation of information from the surrounding environmental conditions is likely important for plants, since it has a direct and major effect on individual plant fitness. This is particularly evident when plants grow under spatially heterogeneous nutrient conditions (Hutchings, M.J., John, E.A., Stewart, 2000; Wacker *et al.*, 2008) or interact with other organisms such as competing plants (Hartnett *et al.*, 1985; Stoll & Schmid, 1998) or mycorrhizal fungi (Bever *et al.*, 2009; Kiers *et al.*, 2011).

As a test case we here compare the responses of single plants and their parts to spatially homogeneous and heterogeneous AMF presence or nutrient supply. Previous studies have demonstrated that under homogeneous conditions plants increase root allocation when nutrient are scarce (Brouwer, 1983; Bloom *et al.*, 1985; Campbell *et al.*, 1991; Reynolds, 1996; Dyer *et al.*, 2001) or when AMF are present (Xu LM., 2010; Zhang et al., 2010; Chapter 3). However, the opposite response can be found under heterogeneous conditions where a single plant produces fewer roots in nutrient-poor patches (Drew 1975, T C Granato 1989, Schmid et al. 1990, Gersani and Sachs 1992, Williamson et al. 2001) or where AMF are absent (Hodge, 2009)than in nutrient-rich patches or where AMF are present. The difference in response between entire plants in homogeneous conditions and plant parts in heterogeneous conditions

suggests a correlative response among the parts of a single plant which in turn indicates that it may integrate environmental heterogeneity at the level of the entire plant (Shemesh *et al.*, 2010). These correlative responses may vary with biotic and abiotic factors such as species identity, competitive conditions or temporal changes of resources (Hodge, 2004, 2009; Shemesh *et al.*, 2010; Mommer *et al.*, 2012).

In the mycorrhizal association, plants invest carbohydrates into fungal partners, which in return provide mineral nutrients (e.g. phosphorus immobilized in complex forms) or other benefits such as protection against biotic (e.g. pathogens and herbivores) and abiotic (e.g. drought) stresses (Parniske, 2008). The majority of plants are capable of establishing mycorrhizal associations with various soil fungi and the most common type of this association is with arbuscular mycorrhizal fungi (AMF) (Smith & Read, 2008). The plant–AMF association has been largely considered as mutualistic, but the net effect on plant fitness ranges from mutualistic to parasitic depending on abiotic (e.g. nutrient availability) and biotic factors (e.g. plant and AMF identity; (Johnson *et al.*, 1997; Kiers & Van Der Heijden, 2006)).

The existence of this range of scenarios explains the importance for the plant to properly integrate information about the abiotic and biotic conditions when associating with AMF. However, it is not known whether plants integrate specific signals for AMF or whether they only respond to the increased phosphate supply created by the AMF; in the latter case, they would just increase carbon flux towards the roots as they do in response to nutrient enrichment (Fitter, 2006). This process could ensure that no hyphae of “cheater” AMF are allowed inside a plant root and only those AMF that provide phosphate are allowed to obtain carbon from the plant host (Hodge, 2009). Therefore, we do not know yet whether a plant could distinguish between nutrient enrichment or AMF presence and modify its response according to

spatial variation in both factors; and if so, whether the response would be localized to individual roots or whether coordination among roots would allow an integrated response of the individual plant.

Previous studies suggest that cooperation between partners in mutualistic associations declines with increasing nutrient availability: when resources are easily accessible, plants do not need "to pay" carbon to AMF because they can obtain nutrients directly via root uptake (Schwartz, 1998; Corkidi *et al.*, 2002). In contrast, at very low nutrient levels, both the fungi and the plants might be nutrient limited, leading to potential competition between AMF and plants (Treseder & Allen, 2002). Again it is not clear what would happen if, in the presence of AMF, parts of the rooting system of an individual plant have easy access to nutrients and other parts not.

To study the potential for correlative responses of single plants to spatially heterogeneous AMF presences or nutrient supply under different conditions we applied different combinations of AMF and nutrient treatments to the two halves of split-root systems of test plants of two contrasting plant species. We measured plant responses at the level of the entire plant as biomass and nitrogen accumulation (referred to as performance) and as biomass allocation (referred to as plasticity) and at the level of the two halves of the root system as belowground biomass. We examined whether the effects of the two factors were additive when they were combined or whether they could substitute (or enhance) each other and thus show significant statistical interaction. Because predictions assumed additive effects (linear increase from AMF in no to one to two compartments and from low-low to low-high to high-high nutrient combinations), differences between observed and predicted values indicated integrated responses, with positive differences indicating positive effects of integration on plant performance. To assess whether root responses were localized

and independent or coordinated and integrated we used split-root systems in which the two root compartments could be exposed to different nutrient and AMF conditions. To account for the possible importance of species identity due to different mycorrhizal dependencies and nutrient requirements, we used two plant species from different functional groups, *Plantago lanceolata* and *Trifolium pratense*. We hypothesized:

1) plant performance increases from low nutrient availability and absence of AMF in both compartments via heterogeneous treatments to high nutrient availability and presence of AMF in both compartments; benefits of AMF presence are higher at low nutrient availability and benefits of high nutrient availability are higher in the absence of AMF;
2) the response of plants in heterogeneous AMF or nutrient conditions cannot be predicted from the response of plants in homogenous conditions because treatment effects are not additive (linear) along a gradient from the least to the most beneficial conditions (increase from AMF in no to one to two compartments and from low-low to low-high to high-high nutrient combinations); positive deviations from linearity indicate positive effects of integration on plant performance, negative deviations indicate negative effects of integration; deviations from additive predictions are strongest when the two root compartments of an individual plant differ in the levels of both rather than only one of the two factors AMF and nutrient availability;

3) the biomass allocation towards roots in AMF- or nutrient-rich compartments increases at the within-plant level but decreases when entire plants are growing in homogeneously AMF- or nutrient-rich conditions;

4) correlative responses to spatial heterogeneity among parts of single plants differ between the type of heterogeneity (AMF presence vs. nutrient availability) and plant species identity.

Material and Methods

Experimental setup

We built split-root systems in which roots from individual plants could be divided between two compartments. Each compartment consisted of two 400 cm^3 square plastic pots stacked inside each other to provide stability and separated by a plastic film to prevent root propagation outside the compartments. To form the split-root system we taped two of these compartments together side by side. To support the stem, the plant was grown with a 3-cm long PVC tube which rested on the soil surface (Figure 1). This design allowed us to separately control the conditions in each compartment of the split-root system. Each compartment received one of four AMF-by-nutrient combinations consisting of factorial combinations of an AMF treatment with the two levels inoculated with AMF ("AMF") and non-mycorrhizal control inoculum ("no AMF") and a nutrient treatment with the two levels "low" and "high": 1= no AMF-low, 2= no AMF-high, 3= AMF-low, 4= AMF-high. These four AMF-by-nutrient combinations could be applied in ten unique ways to the paired split-root systems, yielding ten new treatments. In four of these (1-1, 2-2, 3-3, 4-4), both

compartments received identical AMF-by-nutrient combinations. In the remaining six (1-2, 1-3, 1-4, 2-3, 2-4, 3-4), the two compartments received different AMF-by-nutrient combinations (Table 1). Each of the ten treatments was replicated six times for each of the two test plant species *Plantago lanceolata* and *Trifolium pratense*, giving 120 split-root systems in total.

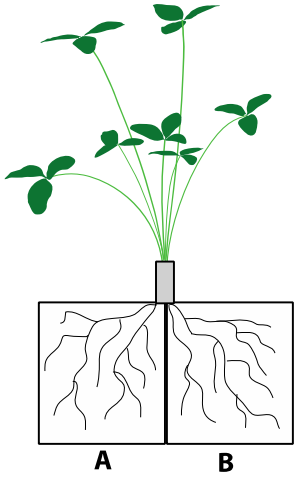

Figure 1. Schematic of the split-root systems used in the experiment with A and B representing the two compartments.

Growth medium

We used a 1:9 volumes of a field-soil:quartz-sand mixture as growth medium. We collected the field soil from a natural grass-clover field at the Agroscope Reckenholz research station in Zurich, Switzerland (47° 25'N, 8°31'E) and sieved it through a 5 mm mesh before mixing. The mixture had a pH of 6.7 and was sterilized with gamma radiation at ca. 50 kGy (range 25–80 kGy LEONI, Aargau, Switzerland).

Table 1. Summary table with the 10 treatments applied in the experiment. These treatments were defined from all the possible paired combinations of both compartments for each split-pot system. Shadowed treatments represent those with identical AMF-by-nutrient combinations for both compartments.

		Compartment B			
	AMF-Nutrient combination	**No AMF-low**	**No AMF-high**	**AMF-low**	**AMF-high**
Compartment A	**No AMF-low**	Treatment 1-1	Treatment 1-2	Treatment 1-3	Treatment 1-4
	No AMF-high	-	Treatment 2-2	Treatment 2-3	Treatment 2-4
	AMF-low	-	-	Treatment 3-3	Treatment 3-4
	AMF-high	-	-	-	Treatment 4-4

Biological material

We obtained seeds of *Plantago lanceolata* L. (ribwort plantain) and *Trifolium pratense* L. (red clover) from local suppliers (FENACO, Switzerland). We surface-sterilized the seeds by soaking them in 5% chloride for ten minutes and afterwards thoroughly rinsed them four times with demineralized water. Seeds were then germinated in sterile sand and individually transplanted as seedlings to single pots for four weeks. Prior to transplanting and to stimulate the outgrowth of lateral roots, we clipped the main root 2 cm below the shoot. After four weeks, we transplanted the seedlings into the split-root systems in the glasshouse. For the mycorrhizal treatment, we used two different inocula: *Funneliformis mosseae* inoculum (isolate JJ964, (Krüger *et al.*, 2012) and a non-mycorrhizal control inoculum, which was propagated as described by Wagg *et al.* (2011). The inoculum from *F. mosseae* had five spores per gram of soil and no spores were observed in the non-mycorrhizal control inoculum. Each AMF compartment received 20 g of inoculum consisting of a mixture

of spores, hyphae, and mycorrhizal root fragments corresponding to approximately 100 spores. For the non-mycorrhizal control, the same amount of inoculum was added in each no-AMF compartment. Because the different inocula might have had different microbial communities, all pots received additionally 5 ml of a standardized microbial wash. We prepared the microbial wash by sieving 25 g of the non-sterilized growth medium with 25 g of both *F. mosseae* and non-mycorrhizal control inoculum in 5 L of distilled water with a series of sieves. To ensure that only bacterial communities could penetrate the wash and to avoid fungal contamination, the finest of these sieves was 10 µm. We placed the pots in a climate controlled growth chamber with a 8:16 h dark:light cycle, a temperature of 16:21ºC day:night and 60% humidity. We watered the pots every other day with tap water and randomly relocated the pots every week.

Nutrient treatment

We supplied the plants with a nutrient solution that was based on the Hoagland solution (Hoagland & Arnon, 1950) but with half of the original phosphorus concentration (6 mM KNO_3; 4mM $Ca(NO_3)_2$; 0,5 mM NH_4NO_3; 1mM $NH_4H_2PO_4$; 1mM $MgSO_4$; 50 µM KCl; 25 µM H_3BO_3; 2 µM $MnSO_4$; 2 µM $ZnSO_4$; 0,5 µM $CuSO_4$; 0,5µM $(NH_4)6Mo7O_{24}$; 20 µM Fe(Na)EDTA). Each compartment of the split-root system received either 2 ml (“low nutrient”) or 6 ml (“high nutrient”) of this nutrient solution every second week. We applied this solution just before watering the plants, to ensure that the nutrients mixed well with the soil.

Harvesting and determination of AMF colonization

We harvested plants after 12 weeks growing in the split-root systems. We removed the growth medium and carefully shook loose the roots from the soil. We washed the plants with tap water, dried them with filter paper and separated the

individuals into roots and shoots. These were then oven-dried at 70º C for 72 hours and weighed. To estimate the percentage of nitrogen in the plant, we ground the shoots and carried out CHN analyses to determine the proportions of major elements by dry combustion (CHN 2000 analyzer, LECO Corporation, USA). To estimate AMF colonization, the dried roots were rehydrated for 12 hours, cleared with 10% KOH and stained with 5% pen-ink vinegar as described in Vierheilig *et al.* (1998). We checked the stained roots for colonization by AMF using the intersect method as outlined in McGonigle *et al.* (1990). For each root sample, we scored 50 line intersections for the presence of hyphae, vesicles, and arbuscules. From these measurements, we estimated the total percentage of root length colonized by AMF.

Plant biomass allocation

We investigated the biomass allocation of plants to roots at two different levels of integration, the entire individual (eq. 1) and the root compartment (e.g. 2). For the first measure, which we call "individual plasticity", we divided the belowground biomass of all roots from the two compartments of a plant by its total biomass. For the second measure, which we call "belowground plasticity", we divided the belowground biomass of all roots from one compartment by the belowground biomass of all roots from the two compartments:

$$individual\ plasticity = \frac{root_a + rootb}{root_a + root_b + shoot} \quad \text{eq. 1}$$

$$belowground\ plasticity = \frac{root_a}{root_a + root_b} \quad \text{eq. 2}$$

Here $root_a$ refers to the belowground biomass in the compartment examined, $root_b$ to the belowground biomass in the neighbour compartment and $shoot$ to the aboveground biomass; all from the same split-root system of an individual plant.

Statistical analyses

All our analyses were carried out using the statistical software R (R version 2.13.0). To meet the assumption of variance homogeneity we used generalized least squares (GLS) models from the "nlme" package of R, which allowed us to use different error variances for *P. lanceolata* and *T. pratense* in models including both species (Zuur *et al.*, 2009), for total biomass, root biomass, shoot-root ratio, AMF colonization and nitrogen content. Only roots with AMF colonization > 0 were included in the corresponding analysis (roots in no-AMF treatments were never colonized by mycorrhiza). For biomass allocation (individual and belowground plasticity), we used beta regression models (Ferrari & Cribari-Neto, 2004) from the "betareg" package. These models assume values in the standard unit interval (e.g. allocation rates or proportions) that are naturally heteroscedastic and easily accommodate asymmetries.

Results

Treatment effects on plant performance: total biomass and nitrogen content

To determine the effects of AMF presence and nutrient addition on plant performance, we first analyzed the variation in biomass and nitrogen content among the four treatments with identical AMF-nutrient combinations in both compartments (see Table 1, main diagonal). For biomass, there was an increase for both plant species along the AMF-nutrient gradient from treatment 1-1 (absence of AMF and low nutrient level in both compartments) to treatment 4-4 (presence of AMF and high nutrient level in both compartments). This implies that the presence of AMF and the increase in nutrients had positive effects on biomass which was confirmed by significant main effects across the four treatments in a factorial model y ~ AMF *

nutrient (R-notation; *P. lanceolata*: $F_{1,19}$=15.84, p<0.001 for the main effect of AMF and $F_{1,19}$=10.70, p=0.004 for the main effect of nutrients; *T. pratense*: $F_{1,20}$=69.61, p<0.001 for the main effect of AMF and $F_{1,20}$=5.90, p=0.025 for the main effect of nutrients). However, the shape and the magnitude of the increase along the gradient were different between the two plant species (Fig. 2). For *P. lanceolata*, high nutrient level had a similarly positive effect as presence of AMF and effects were additive and no interactions were found ($F_{1,19}$=0.51, p=0.483 for the interaction AMF x nutrient), whereas for *T. pratense* high nutrient level in the absence of AMF had no positive effect on biomass ($F_{1,20}$=4.02, p=0.059 for the interaction AMF x nutrient). Furthermore, the increase in biomass along the gradient was larger for *T. pratense* (7-fold) than for *P. lanceolata* (2.5-fold).

Regarding nitrogen in the shoot, results were different when comparing percentages of nitrogen or total nitrogen contents (Fig. 2). For the percentages of nitrogen in the shoot, the presence of AMF had a negative effect in *P. lanceolata* ($F_{1,19}$=22.02, p<0.001) and a positive effect in *T. pratense* ($F_{1,20}$=9.01, p=0.007). However, when focusing on total nitrogen contents, we found positive effects of nutrient availability in *P. lanceolata* ($F_{1,19}$=82.72, p<0.001) and positive effects of both nutrient and AMF in *T. pratense* ($F_{1,20}$=4.03, p=0.058 for nutrient and $F_{1,20}$=58.72, p<0.001 for AMF respectively).

To test whether individual plants could integrate differential effects of AMF presence and nutrient availability between the two root compartments, we compared the observed values from treatments where compartments received a different AMF-by-nutrient combination with those predicted from the mean values of treatments with no variation between compartments. The deviation of the observed from the predicted values was assessed by the confidence interval (calculated as two times the standard

error, Fig. 2) of the first: if it did not overlap with the second, the difference was considered significant. Because predictions assumed additive effects (linear increase from AMF in no to one to two compartments and from low-low to low-high to high-high nutrient combinations), differences between observed and predicted values indicated integrated responses, with positive differences indicating positive effects of integration on plant performance. For example, *P. lanceolata* showed a positive effect of integration on total plant biomass regarding heterogeneous nutrient availability (treatment 1-2: no AMF and low nutrients for one compartment and no AMF and high nutrients for the other compartment), yet this was achieved at the expense of a negative effect of integration on shoot nitrogen concentration (white circles in first two rows and second column of Fig. 2).

Overall, integration seemed to be stronger when the two root compartments of an individual plant differed in the levels of both rather than only one of the two factors, AMF and nutrient availability (columns 6 and 7 vs. columns 2–5 in Fig. 2). Whereas heterogeneous nutrient levels were generally associated with positive integration effects for total plant biomass and total shoot nitrogen, this was not the case for heterogeneous AMF levels. At high nutrient levels, AMF heterogeneity led to negative integration effects in *T. pratense* (column 5 in Fig. 2). Overall, *P. lanceolata* showed more integrated responses than did *T. pratense*.

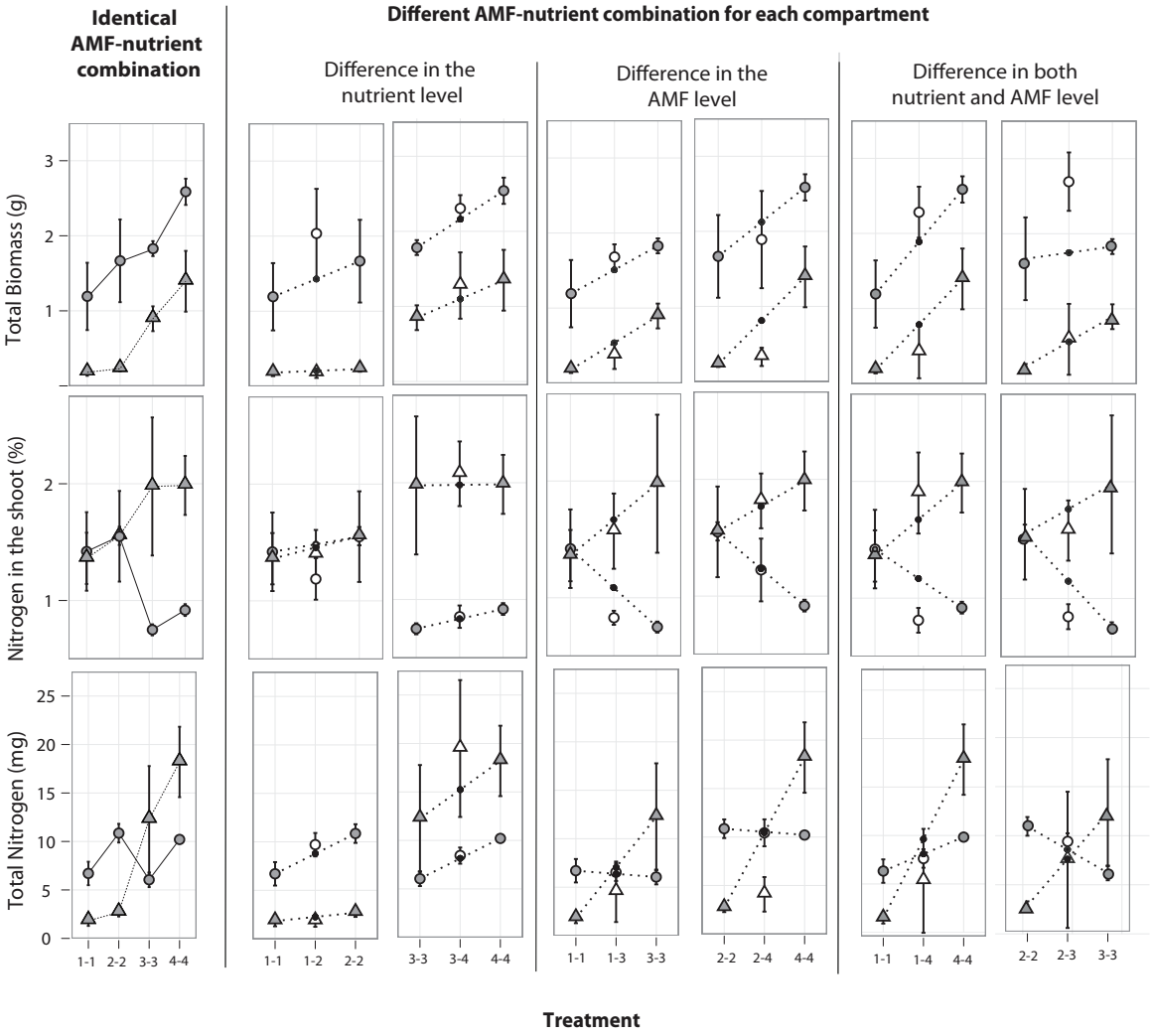

Figure 2. Total plant biomass and nitrogen percentage and total nitrogen in aboveground plant biomass (mean ± 2 SE) for *P. lanceolata* (circles) and *T. pratense* (triangles). First column represents the uniform treatments with no differences in the AMF-by-nutrient combination between compartments. Treatments are plotted from left to right following the AMF-nutrient gradient (represented by a line): no AMF-low (1-1), no AMF-high (2-2), AMF-low (3-3), and AMF-high (4-4). Next six columns represent the six treatments with different AMF-by-nutrient combination in each compartment. Values for these treatments are represented together with a value predicted from the mean of the two corresponding uniform treatments (dashed line with small black-filled circle). Treatments are represented and divided into groups from left to right depending on the type of AMF-by-nutrient combination. Grey filled symbols represent uniform treatments; white open symbols represent values for treatments with variation in the AMF-by-nutrient combinations between compartments.

Effects on belowground biomass at the compartment level

Because our experimental design allowed us to measure root biomass separately under the specific environmental conditions in each compartment of the split-root systems, we also tested whether plant roots responded locally or in a correlated (= integrated) way to treatments. For this we analyzed the effects of the AMF-by-nutrient combination applied to the target compartment and the AMF-by-nutrient combination applied to the neighbor compartment on the root biomass in the target compartment.

The belowground biomass of *P. lanceolata* was on average more than five times as high as the belowground biomass of *T. pratense* (line 2 in Table 2). AMF and increased nutrient treatments applied to the target compartment had significant and positive effects on the belowground biomass in this compartment (lines 3–4 in Table 2). Furthermore, presence of AMF in the neighbor compartment had an additional positive effect on the belowground biomass in the target compartment (line 5 in Table 2), indicating a correlated plant response at the level of the entire root system. All these effects varied between the two species (lines 6–8 in Table 2). Additionally, the belowground biomass of the target compartment was particularly high when AMF were present in the neighbor compartment and the target compartment either also contained AMF or had high nutrient level (interactions in lines 9 or 10, respectively, of Table 2).

Table 2. Summary of significance for terms explaining root biomass for each compartment (analysis of variance output from GLS model).

Term	DF	F	p
Overall mean	1	608.6268	<.0001
Species	1	565.8548	<.0001
AMF treatment	1	169.3322	<.0001
Nutrient treatment	1	6.4852	0.0116
Neighbor AMF treatment	1	22.2017	<.0001
Species x AMF treatment	1	5.8955	0.016
Species x Nutrient treatment	1	6.2324	0.0133
Species x Neighbor AMF treatment	1	5.9726	0.0153
AMF treatment x Neighbor AMF treatment	1	14.3183	0.0002
Nutrient treatment x Neighbor AMF treatment	1	5.1744	0.0239

Individual plasticity

We tested for differences between above- and belowground biomass along the AMF-nutrient gradient and between the two plant species. We found large differences between species: *P. lanceolata* invested nearly twice as much of its biomass in belowground biomass than did *T. pratense* (55.8% vs 31.1%), but the range of variation along the gradient was somewhat larger for *T. pratense* than for *P. lanceolata* (23.9–34.9% vs 51.1–58.2%). Furthermore, for *P. lanceolata* there was no clear pattern along the AMF-nutrient gradient, although allocation to belowground was slightly reduced when the nutrient level was high in both compartments compared with heterogeneous or both low nutrient treatments ($F_{2,50}$=3.86, p=0.027 for main effect of nutrients across all ten treatments). For *T. pratense*, biomass allocation to roots was increased when AMF were present in one of the two compartments rather than none, but reduced again when AMF were present in both compartments ($F_{2,45}$=12.14, p<0.001 for main effect of AMF across all ten treatments). Furthermore,

there was again a trend towards reduced allocation to belowground under increased nutrient levels.

Belowground plasticity

We analyzed the relative belowground biomass allocated to each compartment of the plant. For both plant species, allocation percentages for the two compartments were equal when they both received the same AMF-by-nutrient combination (main diagonal in Table 1 and four rows at bottom of Fig. 3). The largest difference in allocation percentages between compartments was found in treatment 1-4 where one compartment had no AMF and low nutrient level and the other did have AMF and high nutrient level; here allocation percentages were strongly biased towards the compartment with presence of AMF and high nutrient levels (second row in Fig. 3). The differential between the two sides in treatment 1-4 was larger for *T. pratense* than for *P. lanceolata* (17.3%–82.7% vs. 31.9%–69.1%). When only the nutrient level differed between compartments, we found preferential allocation to the compartment with higher nutrient level for *P. lanceolata* when AMF was present in both compartments (rows 5–6 in Fig. 3).

In general, plants allocated more biomass to the compartments with presence of AMF, but when the nutrient level was high for both compartments, this preferential allocation was slightly reduced for *P. lanceolata* and not found any more for *T. pratense* (rows 3–4 in Fig. 3). Finally, when high nutrients were offered in one and AMF in the other compartment there was a preferential allocation for the compartment with AMF, in particular in *T. pratense* (row 2 in Fig. 3).

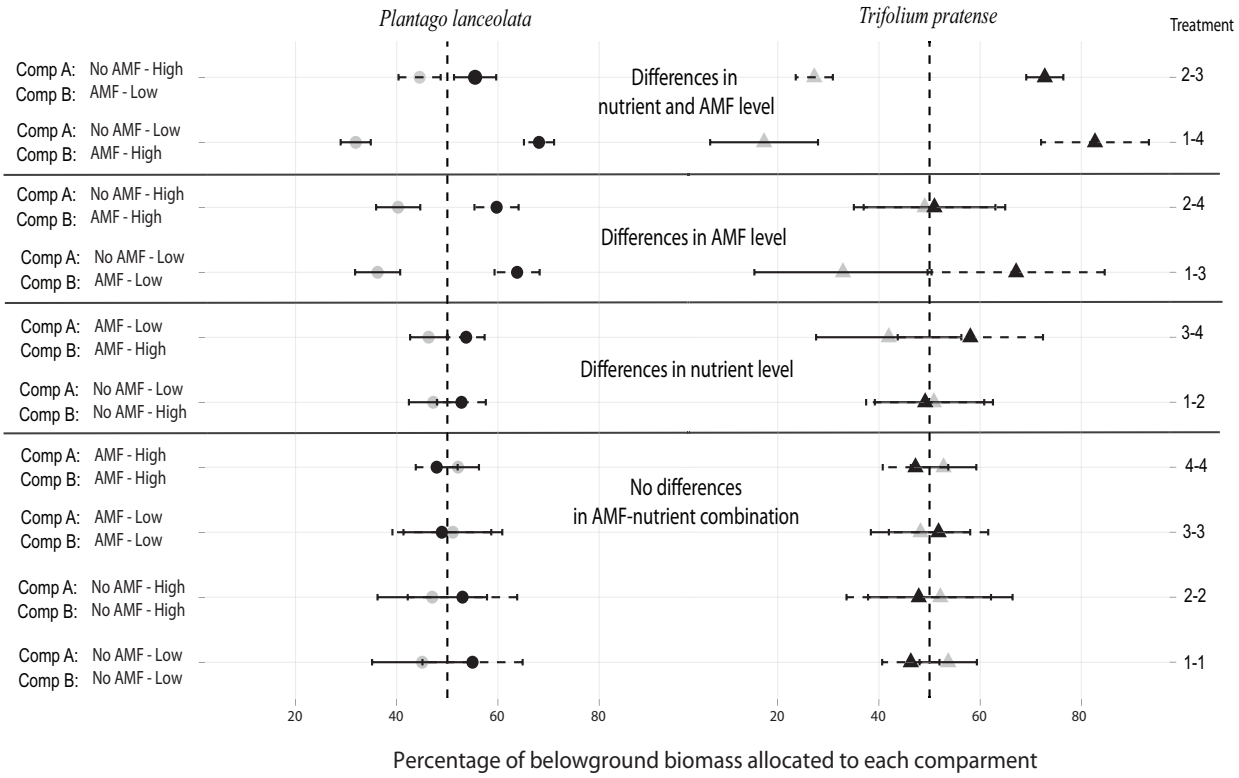

Figure 3. Percentage of belowground biomass (mean ± 2 SE) allocated to each compartment (grey symbols: compartment A; black symbols compartment B) for every treatment. Treatments are represented and divided into groups from bottom to top depending on the type of AMF-by-nutrient combination. Circles represent *P. lanceolata* and triangles represent *T. pratense*. Dashed lines indicate equal proportions of 50% of belowground biomass in the two compartments.

Effects on mycorrhizal colonization in compartments with AMF

Large differences were found between species, with *T. pratense* having on average nearly twice as much of its root length colonized by AMF than *P. lanceolata* (42.5% vs 24.3%; $F_{1,105}$=63.49, p<0.001). For both plant species, the percentage root colonized was reduced when either of the compartments had high nutrient level and increased when both had low or both had high nutrient level ($F_{1,105}$=4.38, p=0.039 for interaction nutrient treatment x neighbor nutrient treatment, main effects were not

significant). *Trifolium pratense* had also significantly lower infection values when the neighbor compartment had no AMF ($F_{1,105}$=4.07, p=0.046).

Effects of mycorrhizal colonization on plant biomass

We used those plants with AMF in both root halves to test if there was a correlation between total biomass and mycorrhizal colonization: for *T. pratense* a positive but curvilinear relationship was found (quadratic polynomial: R^2=0.30, p=0.003), whereas for *P. lanceolata* there was no significant correlation (Fig. 4).

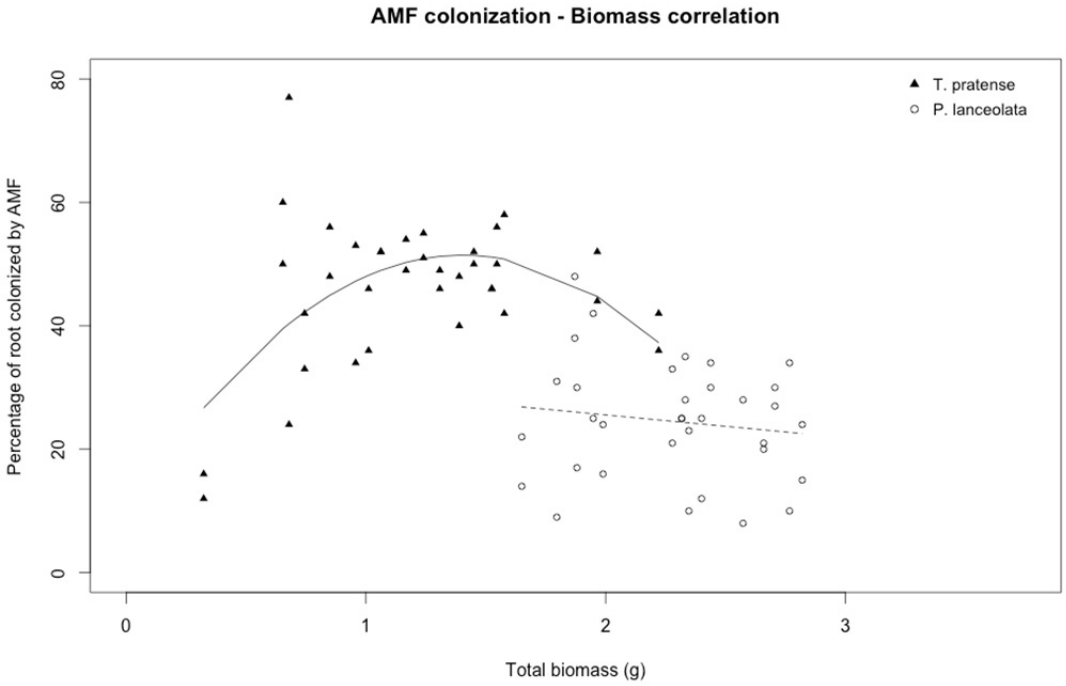

Figure 4. Correlation between total biomass and percentage of root colonized by AMF for *P. lanceolata* and *T. pratense.*

Discussion

Using split-root systems, we found correlative responses between the two halves of root systems of single plants exposed to spatial heterogeneity in AMF presence or nutrient availability, suggesting that plants can integrate signals coming

from different parts. In particular, biomass allocation towards roots in AMF- or nutrient-rich patches was increased at the within-plant level, which was in contrast to the decreased allocation of biomass to roots when entire plants were growing in homogeneously AMF- or nutrient-rich conditions. One species, *Plantago lanceolata*, was able to modify its response to either AMF or nutrient at any level while for the other, *T. pratense*, the presence of AMF seemed to be essential.

Hypothesis 1: beneficial effects of AMF and nutrients under homogeneous conditions

Our first hypothesis was that AMF presence and nutrient supply were actually beneficial to the test plants and that this should be seen when plants were growing under homogeneous conditions. Our findings confirmed that spatially uniform AMF presence and nutrient availability positively affect plant performance in terms of total biomass accumulation, with highest performance in treatment combinations where both AMF are present and nutrient availability is high (treatment 4-4).

However, we could not confirm that benefits of AMF presence were higher at low nutrient availability and benefits of high nutrient availability were higher in the absence of AMF. Instead, *P. lanceolata* was equally and additively affected by AMF presence and nutrient availability, while *T. pratense* was more strongly affected by AMF presence than by nutrient availability, which only had a positive effect in the presence of AMF. Effects of environmental treatments on nitrogen content in the shoot varied depending on the measure used. Percentage values were mainly affected by the presence of AMF: in absence of AMF there were no differences between plant species but in the presence of AMF *T. pratense* showed increased and *P. lanceolata* reduced values. Conversely, total nitrogen content was mainly and positively affected

by nutrient availability in *P. lanceolata* while *T. pratense* again showed a positive effect of AMF presence.

These differences between the two test plant species in homogeneous conditions may be due to the fact that the legume *T. pratense* associates not only with AMF but also with nitrogen-fixing *Rhizobia*. This tripartite symbiotic association (AMF–legume–*Rhizobia*) is known for positive synergistic interactions among its members—with improved rates of phosphorus uptake, nitrogen-fixation and plant biomass—under conditions of reduced nitrogen and phosphorus inputs (Azcón-Aguilar & Barea, 1992; Xavier & Germida, 2002; Jia *et al.*, 2004; Chalk *et al.*, 2006). Under low nitrogen and phosphorus inputs, the major factor limiting nitrogen-fixation is usually soil phosphorus availability (Toro, Azcón & Barea, 1998) and in absence of AMF supplementary phosphorus fertilization is generally necessary to maintain appropriate nitrogen-fixation rates (Andrade, 1998). Therefore, the addition of AMF into the system would mitigate not only phosphorus but any other nutrient limitation in the plant that might be restrictive for *Rhizobia* (Pacovsky *et al.*, 1986; O'Hara, 1998; Chalk *et al.*, 2006) and, as shown in our study, produce a strong positive effect on plant performance. Since under very low nitrogen availability AMF should be also limited, reducing their symbiotic performance, the improvement in the *Rhizobia* symbiosis would also favour the AMF and its symbiosis with the plant partner, explaining the positive synergistic interaction between AMF presence and nutrient availability mentioned above.

On the other hand, for *P. lanceolata*, there is no "extra input" of nitrogen into the system, and although a large part of the reduction in the nitrogen percentages in the shoot could also be explained by the decrease in nitrogen levels (Treatment 3-3, Fig.2) or the increase in biomass (treatment 4-4, Fig.2), the presence of AMF

especially in the low nutrient levels, might have resulted in competition with the AMF for the limited nitrogen (Treseder & Allen, 2002). Because AMF are more efficient scavengers for nutrients from the soil than are plant roots, the threshold for nutrient limitation may be lower for mycorrhizal fungi than for plants (Allen, 1991), which could explain not only the reduction in nitrogen percentage in presence of AMF but also the increase in biomass for *P. lanceolata* found under higher nutrient levels.

The different degree of benefit from AMF for the two plant species was also reflected by different AMF colonization levels and their correlation with plant biomass: while for *T. pratense* biomass increased with higher colonization levels (except for highest biomass values), in *P. lanceolata* biomass was slightly negatively correlated with colonization levels.

Hypothesis 2: integrative effects of AMF or nutrients under heterogeneous conditions

We expected that the response of plants to spatially heterogeneous treatments in the two halves of plant root systems could not be predicted from the response of plants in homogenous conditions because treatment effects would not be additive along a gradient from the least to the most beneficial conditions (increase from AMF in no to one to two compartments and from low-low to low-high to high-high nutrient combinations).

Using split-root systems, we showed that plant responses were indeed not predictable based on responses to spatially uniformly applied treatments when the heterogeneity was in either nutrient availability or AMF presence, which suggests a correlative and thus integrative response to AMF and nutrient heterogeneity. The strongest deviation from predictions based on uniform, independent responses

occurred in cases where spatial heterogeneity of AMF presence and nutrient availability were combined (treatments 1-4, 2-3).

Furthermore, we found interactive effects between spatial heterogeneity in the two environmental factors and plant species identity. For *T. pratense,* positive effects caused by the presence of AMF in one of the compartments (Treatment 1-3 and 2-4, Fig. 2) were lower than expected, even under high nutrient levels. This can be interpreted as negative effect of integration and might be explained by the high mycorrhizal dependency of legumes (Scheublin *et al.*, 2007): when AMF were present in only one compartment it might have not been enough to fulfil the plant's demand on nutrients, especially regarding phosphorus. For the plant, in this case the costs of maintaining the AMF on one side of the root system may have exceeded the benefits; an idea which is also supported by the lower AMF infection values found when the neighbour compartment had no AMF. For *P. lanceolata,* conversely, the increase in biomass when AMF was present in just one of the compartments was actually higher than expected for most of those treatments, and especially higher when nutrient level was low in the compartment with AMF and high in the compartment with absence of AMF (Treatment 2-3). This positive effect of integration indicates that for this plant species the benefits of maintaining the AMF on only one side of the root system must have exceeded the costs, especially under low nutrient levels. When AMF occur on both sides, the association must become more costly for the plant, perhaps due to competition for nitrogen as suggested above. *Plantago lanceolata* has been reported to have good root foraging strategies to access to nutrient patches (Kembel, 2005), which explains its lower mycorrhizal dependency and its higher values in nitrogen content in the absence of AMF.

In our study we further investigated whether plant roots responded locally or in a correlated (= integrated) way to the AMF and nutrient treatments. Consequently we analyzed the effects of the AMF-by-nutrient combination applied to the target compartment and the AMF-by-nutrient combination applied to the neighbor compartment on the root biomass in the target compartment. Besides the positive effect of AMF presence and nutrient increase on the biomass of the target compartment, AMF presence and nutrient increase (this last one only when AMF was present in the target compartment) in the neighbor compartment had a further positive effect on the root biomass of the target compartment. Thus, because the effects of the treatments reached beyond a single compartment we consider our results as an indication of a correlated plant response at the level of the entire root system, although again responses varied between the two plant species.

Hypothesis 3: correlative biomass allocation to plant roots under heterogeneous conditions

We expected biomass allocation towards roots in AMF- or nutrient-rich patches to be increased at the within-plant level but decreased when entire plants were growing in homogeneously AMF- or nutrient-rich conditions. Our results confirmed that *P. lanceolata* showed a plastically increased biomass allocation to roots under spatially uniform low nutrient avalability but not in the absence of AMF. For *T. pratense*, there was a trend to reduce biomass allocation to roots under increased nutrient levels but in this case it was AMF presence in both compartments that significantly reduced allocation to belowground biomass. Under heterogeneous conditions, as expected, root biomass allocation was strongly biased towards the compartment with presence of AMF or high nutrient levels. This can be seen as an integrated response because in homogeneous conditions entire plants should (and did)

show the opposite plasticity response, namely reduced allocation of biomass to the organs with plentiful resources (here soil nutrients and presence of AMF).

Hypothesis 4: differences between type of heterogeneity and plant species

We hypothesized that the correlative responses to spatial heterogeneity among parts of single plants would differ between the type of heterogeneity (AMF presence vs. nutrient availability) and plant species identity. Our results showed indeed larger differentials between sides for *T. pratense* than for *P. lanceolata* (17.3%–82.7% vs. 31.9%–69.1%) that might be explained by the interactive effects of AMF presence and increased nutrient levels already seen with the legume. Also, and similar to the positive integration at the total plant level, only *P. lanceolata* showed preferential allocation to the compartment with higher nutrient level, but interestingly this was only found when AMF was present in both compartments.

Regarding differences in the AMF distribution, in general plants allocated more biomass to the compartments with AMF, but under high nutrient levels this differential was reduced for *P. lanceolata* and disappeared for *T. pratense*. This might be seen as a simultaneous integrative response of the plant at both belowground level (preferential allocation to the compartment with AMF) and individual level (reduced allocation to belowground biomass under high nutrient conditions). The preferential allocation towards AMF found when offered in one compartment and high nutrient in the other, might be an indication of specific AMF recognition by the plant: when nutrients come from an AMF they have to invest in return-payment whereas they would not do it with abiotic nutrients.

Hence, our results show how belowground plasticity in biomass allocation to differently treated root halves is opposite to the individual plasticity in biomass

allocation of whole plants with regard to both the nutrient and the AMF treatment. Plasticity in response to within-plant spatial heterogeneity in nutrient availability occurs as roots forage for patchily distributed nutrients within the soil (Hodge, 2006) and similar results have also been for AMF. Although the plastic response was stronger in *T. pratense*, *P. lanceolata* seemed to be more precise it its response and integration, preferring in nearly every treatment (except in Treatment 1-2) the compartment with more beneficial conditions (e.g. more increased allocation to roots with AMF under uniformly low levels of nutrients than under uniformly high level of nutrients).

Responsiveness to resource gradients requires the integration of environmental information (Aphalo & Ballare, 1995). According to their relative adaptive values, plants are able to compare patches and discriminate between them by mean of various tropic and nastic movements (Karban, 2008; Shemesh *et al.*, 2010). In our study, plants were able to compare treatments in each compartment, discriminate between AMF and nutrient availability at different levels and show integration, but with different values of accuracy for each plant species. As *P. lanceolata* and *T. pratense* differ in their mycorrhizal dependency and root foraging strategies these differences may be related to the different strengths of integration and plastic responses along our treatment-combinations gradient.

Our results suggest that plants perceive AMF presence and nutrient availability as different signals and are able to show integrated responses accordingly, both at the level of the individual plant and at the root system. This study, although carried out under artificial controlled conditions, attempts to account for the "relative influence" of each factor (Klironomos *et al.*, 2011) and confirms the abilities of plants to respond in an integrated way to AMF and nutrient availability. The integrated

responses of the plant to AMF presence and nutrient availability appear to be context dependent, dissimilar for different plant species and controlled by a correlative behaviour of plant parts (i.e. plasticity in the allocation to above and belowground biomass) rather than by a central control exerted through an organ such as the central nervous system of animals.

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Chapter Four

Enhanced carbon allocation to arbuscular mycorrhizal fungi positively affects phosphorous transfer to the plant

Alicia Argüello, Michael J. O'Brien, Marcel van der Heijden, Andres Wiemken, Bernhard Schmid and Pascal A. Niklaus

Abstract

The net outcome of the symbiosis between plants and Arbuscular Mycorrhizal Fungi (AMF) can range from positive to negative depending on the ecological context and partners identity. Despite it is unclear how the mutualism is maintained, preferential allocation to more cooperative partners and bidirectional control of the resource exchange (trade) have been recently pointed out as the main likely processes to stabilize the cooperation for this ancient symbiosis. In this study, we combined the use of radioactive isotopes (^{14}C and ^{33}P) and compartmentalization of model systems, to test *in vivo* for preferential allocation and reciprocal rewards in the AMF–plant symbiosis. We used two plant species differing in their mycorrhizal dependencies (*Plantago lanceolata* and *Trifolium pratense*) and two different AMF isolates varying in their beneficial effects on plants (*Rhizophagus irregularis* and *Funneliformis mosseae*), which enables us to use natural variation in the plant–AMF trade. We confirmed higher allocation to the more cooperative AMF (*R. irregularis*) and reduced allocation to the less cooperative AMF (*F. mosseae*) when the choice of *R. irregularis* was present. Both AMF partners seemed to be better quality symbionts with *T. pratense* than with *P. lanceolata*, but the first plant species also allocated relatively more carbon to the AMF partners than *P. lanceolata*. Additionally, the carbon costs per unit of phosphorous transferred to the plant were always higher for *P. lanceolata* than for *T. pratense* and especially when associating with the less cooperative *F. mosseae*. Although further studies across a greater diversity of symbionts and environments are needed, our study shows that reciprocal rewards *in viv*o from both partners enhanced the mutualism and can clarify the evolutionary persistence of the plant–AMF symbiosis.

Key words: AMF–plant symbiosis, cooperation, mutualism, partner identity, preferential allocation, reciprocal rewards, trade.

Introduction

Mutualistic interactions among organisms are widespread and in the case of the plant–arbuscular mycorhizal fungi (AMF) interaction have evolved over 400 million years ago. Nevertheless, evolutionary theory suggests that selection should favour "cheating" partners that deliver less benefit over those cooperative partners that reciprocate (Kiers & Van Der Heijden, 2006; Bever *et al.*, 2009) and this would threaten the persistence of the mutualistic plant–AMF interaction. Consequently, the mechanisms promoting this association remain unclear and explaining its persistence through evolutionary time continues to challenge ecologists and evolutionary biologists (Sachs *et al.*, 2004; Gardner *et al.*, 2007; Grman *et al.*, 2012; Verbruggen *et al.*, 2012).

Plants can modify their resource allocation as a response to changes in environmental conditions (e.g. nutrient availability, Chapter 3). However, it is unclear whether plants can also discriminate between different AMF species or genotypes and preferentially allocate resources to more beneficial partners (Helgason *et al.*, 2002; Kiers & Van Der Heijden, 2006; Fitter, 2006; Bever *et al.*, 2009; Kiers *et al.*, 2011). Partner choice has already been investigated in other mutualistic interactions, for example in those between legumes and rhizobia where "host sanctions" (Denison, 2000) by the plant against less-effective rhizobial partners were found. As in the case of a legume plant which can be infected by multiple strains of rhizobia, most vascular plants are typically host to multiple species or genotypes of AMF (Vandenkoornhuyse *et al.*, 2002; Scheublin *et al.*, 2004). Thus, sanctions against poor-quality fungal partners may be involved in enforcement mechanisms that stabilize the mutualistic interaction (Kiers & Van Der Heijden, 2006).

Recently, preferential allocation of plant photosynthetic carbon to the more mutualistic partner between two AMF species was shown (Bever *et al.*, 2009; Kiers *et al.*, 2011). Preferential allocation of plant carbon to the more beneficial AMF at the scale of parts of the

root system (or even of individual rootlets) might enhance the fitness of "intelligent" plants and select for cooperative AMF, thus contributing to the stabilization of mutualistic plant–AMF interactions. However, there is little empirical support for such decision-making in plants (Bever *et al.*, 2009; Kiers *et al.*, 2011; Verbruggen *et al.*, 2012).

Recent work using *in vitro* root organ cultures further demonstrated that reciprocal provisioning (which could be compared to fertilizer effects) occurs between plant and AMF partners. In this case, a plant individual provides more carbon to that AMF partner that contributes more phosphorus and, reciprocally, an AMF individual provides more phosphorous to that plant partner that contributes more carbon (Kiers *et al.*, 2011). These authors view this reciprocal provisioning as a biological market (Noë & Hammerstein, 1995; Schwartz & Hoeksema, 1998) where both partners have a role in controlling the exchange of resources and therefore stabilize the mutualistic interaction. No comparable mechanisms have so far been reported for other mutualistic interactions, and indeed no other mutualistic interactions seem to have persisted for as long through evolutionary time scales as did the one between plants and AMF.

In the present study, we used microcosms with compartmentalized plant root systems to study plant carbon allocation and phosphorus acquisition in relation to multiple AMF species offered as partners. Dual C- and P-isotope labeling allowed us to test decision-making processes in plants in the AMF symbiosis, i.e. allocating more C to the AMF symbiont returning the largest amount of P per unit of C invested. We used two plant species differing in their mycorrhizal dependencies (*Plantago lanceolata* < *Trifolium pratense*), in combination with two different AMF species (*Rhizophagus irregularis* and *Funneliformis mosseae*) that we previously found to vary in their beneficial effects for these two plant species (Chapter 1). A non-mycorrhizal treatment allowed us to test whether beneficial effects on individual plant performance (e.g. biomass, shoot:root ratio, phosphorus concentration) depended on AMF

presence and identity of both AMF and plant. The presence of decision-making processes in plants was assessed by testing whether a plant individual could adjust root biomass or C allocation between heterogeneous treatments (e.g. different AMF partners), each occupying one of the two separated halves of the root system. Furthermore, we asked whether plants show any partner selective response (i.e. allocating more biomass or carbon) depending on the specific AMF performance (i.e. phosphorous transport to the plant and carbon cost derived from the symbiosis) and whether plant services (carbon allocation to the AMF) would also affect the cooperative behavior of the AMF partners.

Material and Methods

Design of split-root systems

We grew host plants in microcosms dividing their root systems into two halves by means of a PVC separation (Fig. 1). Each side of the split-root system was inoculated with one of the two AMF species or left un-inoculated. Both root-system halves were further subdivided into three 200 mL compartments at increasing distance from the plant center (0–2 cm, 2–4 cm, 4–6 cm) by 20-µm nylon mesh. This mesh allowed hyphae but no roots to pass through. Roots could therefore only colonize the central two compartments, whereas associated AMF could colonize all three compartments of a side; but AMF of the two sides of the split-root system were kept separate. In the course of the study, radio-phosphorus was added to one of the outermost compartments to quantify plant phosphorus acquisition through the respective AMF partner. Roots could not access radio-phosphorus directly because there was a root-free middle compartment serving as separation between the roots and the soil to which the radio-label was added. At the same time, plants were labeled with $^{14}CO_2$, allowing the quantification of C supply to the respective side of the root system and its associated AMF. This setup allowed us to quantify plant C investment to the root and its AMF partner relative to P acquired from the AMF partner occupying the half with the labeled compartment.

Plant and AMF species

Field-soil from a natural grass-clover field (pH 6.7, Agroscope Reckenholz research station, Zürich, 47° 25'N, 8°31'E) was sieved (5-mm mesh) and mixed with quartz-sand (1:9 v/v). The mixture was sterilized by gamma irradiation (25–80 kGy, LEONI, Aargau, Switzerland) and filled into the central four microcosm compartments. The outermost compartments were filled with the same mixture, but only when the radio-phosphorus label was applied. Until then, they were filled with a polystyrene spacer to reduce desiccation and prevent algal growth.

Seeds of *Plantago lanceolata* L. (ribwort plantain) and *Trifolium pratense* L. (red clover) (FENACO, Switzerland) were surface-sterilized by soaking in 5% aqueous hypochlorite solution for ten minutes. Then, they were rinsed four times with demineralized water and germinated in sterile sand. The emerging seedlings were transplanted to individual pots and grown for four weeks until they were transplanted to the microcosms. While transplanting, the main roots were clipped 2 cm below the shoot to promote lateral root growth. Once in the microcosms, plant individuals were supported with a 3-cm long quartz sand-filled PVC tube section resting on the soil.

Each of the two middle compartments was amended with one of three inocula: *Funneliformis mosseae* ("M"; previously named *Glomus mosseae;* Krüger *et al.*, 2012), *Rhizophagus irregularis* ("I"; previously named *Glomus intraradices;* Krüger *et al.*, 2012) or remained AMF-free ("C"). All AMF combinations were realized, resulting in six different AMF treatments (CC, CI, CM, II, IM and MM) per plant species. As the different AMF inocula might have contained different bacterial communities, all pots also received 5 mL of a microbial wash. This wash was made by filtering (10 μm pore size) five liters of a suspension prepared from 25 g of the soil mixture and 25 g of each AMF inoculum. We further applied

2.5 ml of rhizobium solution (OD580 of 0.2; *Rhizobium trifolii*, DSM 6040) to ensure adequate nodulation. All harvested roots of *Trifolium* had active nodules.

Growth conditions

The microcosms were placed in a climate-controlled growth chamber with a 16/8 h light/dark cycle, a temperature of 21/16°C (day/night), 60% relative humidity and an average photon flux density of 400 PE m^{-2} s^{-1} in the photosynthetically active range. The pots were watered every other day with deionized water and every week their positions were randomly changed to ensure equal growth conditions for all the pots.

Plants were supplied with a nutrient solution that was based on the Hoagland solution (Hoagland & Arnon, 1950) with half of the normal P concentration (6 mM KNO_3; 4mM $Ca(NO_3)_2$; 0,5 mM NH_4NO_3; 1mM $NH_4H_2PO_4$; 1mM $MgSO_4$; 50 µM KCl; 25 µM H_3BO_3; 2 µM $MnSO_4$; 2 µM $ZnSO_4$; 0,5 µM $CuSO_4$; 0,5µM $(NH_4)6Mo7O_{24}$; 20 µM Fe(Na)EDTA). Each root compartment of the split-root system received 2 ml of this nutrient solution every second week. This solution was applied just before the plants were watered, to make sure that the nutrients mixed well with the soil.

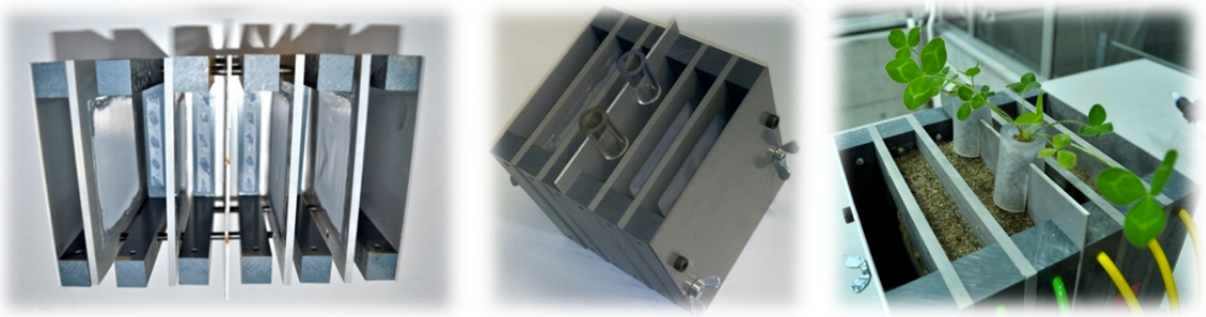

Figure 1. Split-root system. From left to right: a split-root system in which the different compartments can be distinguished, the complete split root-system fitted together, a split-root system with two *Trifolium pratense* seedlings.

^{14}C and ^{33}P labeling

After 10 weeks, the polystyrene space holder was removed from the outermost compartments and 37 MBq of radio-phosphorus ($H_3{}^{33}PO_4$) mixed with substrate filled into the compartment. The same procedure was applied to the other side, but using non-radioactive phosphorous (^{31}P). Two weeks after ^{33}P labeling, plant individuals were pulse-labeled with $^{14}CO_2$ in a transparent Plexiglas chamber (Fig 2). Throughout the labeling, the CO_2 concentration was monitored with an infrared gas analyzer (LiCOR 6200, LiCOR, Nebraska) and maintained above 300 ppm by successively releasing $^{14}CO2$ from a sodium bicarbonate ($NaH^{14}CO_3$) solution by adding 5% H_2SO_4 with a syringe. The air within the chamber was mixed with a fan. Excessive heating of the chamber was prevented by cooling with a heat exchanger connected to an ice:water mixture.

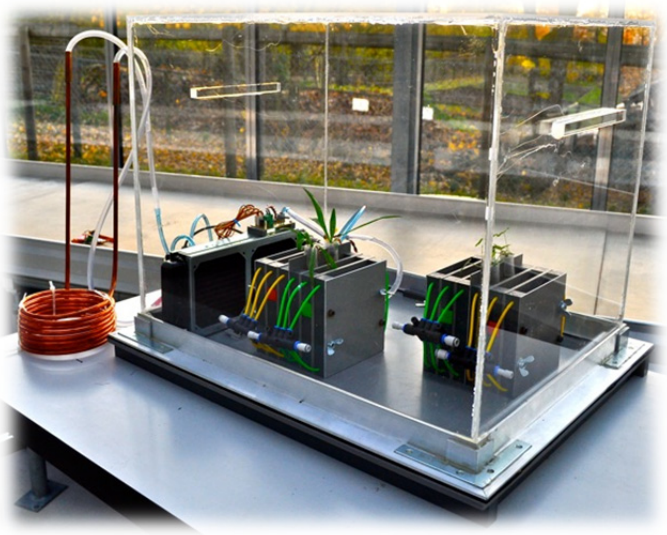

Figure 2. Plexiglas chamber built for ^{14}C labeling.

After one photoperiod, the microcosms were removed from the chamber. Both sides of the microcosms were covered with Parafilm and soil respiration trapped separately by extracting air from the headspace of the compartments with a peristaltic pump and trapping the CO_2 contained in 30 ml traps filled with 1M NaOH (Fig 3). After 24, 48, 72, 96 and 112 hours, 1 mL aliquots were collected from each trap and analyzed for ^{14}C (see below).

Figure 3. System for trapping the soil respiration fraction. From left to right: split-root system with the tubing from the three compartments on each side merging into a single flux; the flux from each side was coupled to one channel from the peristaltic pump; each of the channels was connected to a washing bottle where CO_2 was trapped.

Destructive harvest

Microcosms were harvested 112 hours after C-label application. After shoots had been harvested, roots were collected from both sides of the microcosms separately. Soil was recovered from all six compartments separately.

Fresh subsamples from shoots, soil and roots were combusted in a muffle oven (12 hours at 600ºC) and the ash dissolved in 2 mL 5.6 M HCl, followed by 5 mL H_2O. 1 mL of this solution was mixed with 4 ml cocktail (Ultima Gold, Perkin Elmer, The Netherlands) and ^{33}P activity recorded by liquid scintillation counting (TRICARB 2900 TR, Packard, USA). Another 1 ml aliquot was used to determine total phosphorus concentration (San^{++} continuous flow analyser, Skalar Analytical,The Netherlands).

A second subsample of the root and shoot material was dried (70 ºC, 72 h) and re-weighed. These samples were dry-combusted in a sample oxidizer Packard Sample Oxidizer Model 307 (Hewlett Packard, USA) evolving $^{14}CO_2$ trapped in 10 ml Carbosorb (Perkin Elmer, The Netherlands), 10 ml Permafluor (Perkin Elmer, The Netherlands) cocktail added,

and ^{14}C activity determined by liquid scintillation counting. ^{14}C in soil samples was determined by the same procedure.

AMF colonization

Root subsamples were cleared with 10% KOH, followed by staining with 5% pen-ink vinegar mixture as described in Vierheilig *et al.* (1998). Stained roots were scored for the presence of AMF colonization using the intersect method outlined in McGonigle *et al.* (1990). For each sample, 50 line intersections per root sample were scored for the presence of hyphae, vesicles, and arbuscules. From these measurements the total percentage of root length colonized by AMF (which equals the amount of root length occupied by hyphae) was estimated. We found signs of AMF colonization in all our inoculated pots and in some of our AMF-free sides from the heterogeneous AMF-combined treatments. The latter were removed from all our analyses except for total biomass and shoot-root ratios.

Experimental design

In total, our experiment encompassed 96 microcosms (2 plant species × 6 AMF combinations × 2 ^{33}P-labelled root system sides × 4 replicates). Our experiment was organized in sets of six microcosms (groups), since these fitted into the labeling chamber. A group contained one plant species with all six AMF combinations. A total of 16 groups were processed sequentially over a period of six weeks. Two consecutive groups formed a block, which were identical in terms of plant and AMF species but the radio-phosphorus label was applied to opposite sides of the microcosms.

Statistical analyses

Total plant biomass and shoot:root ratio were analysed as a function of plant species identity, AMF combination and their interaction. We used a generalized least-squares analysis (GLS) with a weighted variance to account for heteroscedasticity for both independent

variables. The percentage of ^{33}P transferred to the shoot was analysed as a function of plant species identity, the AMF identity of the side with ^{33}P addition, the AMF identity of the side with ^{31}P addition and their interactions with a linear mixed effects model. AMF colonization was modelled with a GLS as a function of plant species and AMF identity.

Root biomass allocation was analysed for each side of the split root as the proportion of total root biomass, and plant species identity, AMF treatment in the side analysed, AMF treatment on the opposite side and all two-way interactions between those three variables were used as explanatory variables. We analysed the effect of plant species identity and AMF treatment on the concentration of ^{14}C per mg of root biomass (Bq mg^{-1}) as a function of plant species identity, AMF treatment in the side analysed, AMF treatment on the opposite side and the interaction between the two AMF sides with a linear mixed effects model. The amount of ^{14}C in the hyphal compartment (Bq) was analysed as well as a function of plant species identity, AMF treatment in the side analysed, AMF treatment on the opposite side and the interaction between the two AMF sides. We again used linear mixed effects models for this variable in order to account for known error. In order to assess the costs of the mutualism, we calculated the ratio of C costs to P transferred (total ^{14}C (Bq) allocated to root and hyphal compartment divided by total ^{33}P (Bq) transferred to plant shoot) for the four plant-AMF combinations (*P. lanceolata*/*F. mosseae*, *P. lanceolata*/*R. irregularis*, *T. pratense*/*F. mosseae*, and *T. pratense*/*R. irregularis*). We analysed this ratio as a function of plant species identity, the AMF identity of the side with ^{33}P addition, AMF treatment on the opposite side and their interaction. We log transformed the data to meet the assumptions of linearity and used a GLS with a separate variance structure for each plant species.

A random effect (group within block) to account for variation in time and space with the addition and sampling of ^{33}P (block) and ^{14}C (group) was used in all linear mixed effects models. Furthermore, a weighted variance, which allowed unequal variance for each plant

species, was used in all linear mixed models. All analysis was performed with the nlme package (Pinheiro &Bates 2001) in the R statistical software (version 3.0.1). The gls and lme functions in this package allowed us define random effects and define different variance structures for *P. lanceolata* and *T. pratense* (Zuur *et al.*, 2009). However, due to the complexity of the design and limitations of the lme function we could not break down the random model into all its possible components. As a consequence, some of the statistical tests as well as the calculated confidence limits must be considered as too liberal or narrow, respectively, for the comparison between aggregated treatments of treatment combinations.

Results

We present the results in two separate sections: first, we show the plant responses to AMF treatments as total biomass (shoot plus roots from both compartments), shoot:root ratios, AMF colonization and the percentage of the total ^{33}P applied in the labeling compartment transferred via AMF to shoots. Secondly, we focus on the plant decision-making processes by assessing changes in the root biomass allocation to each compartment, ^{14}C concentration in roots, total ^{14}C in the hyphal compartment and the carbon costs (total carbon allocated to root plus hyphal compartments) per unit of phosphorous transfer to the plant shoot.

Plant responses to AMF treatments

Plant biomass

AMF infection significantly increased total biomass (shoots plus roots) of both plant species (Fig. 1; $F_{5,83}$=250.72, P <0.001), independent of AMF identity. Plant species differed in their response to the combined AMF treatment ($F_{5,83}$=7.46, p = <0.001), an effect largely

driven by lower biomass in *T. pratense* with no AMF applied or AMF present only on one side of the root system.

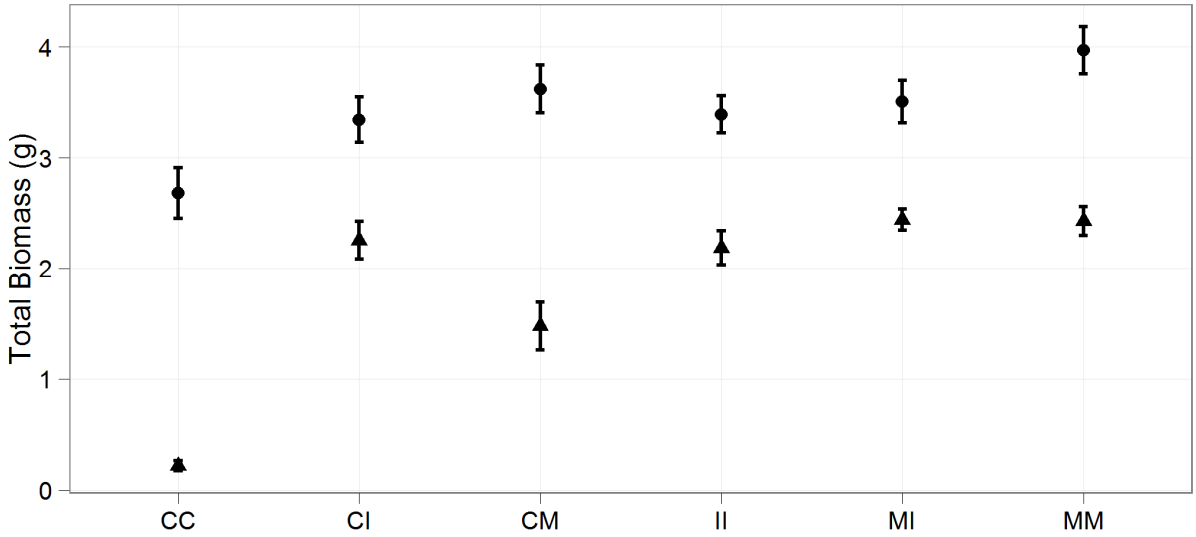

Figure 4. Total biomass (roots plus shoots; mean ± 1 SE) for *P. lanceolata* (circles) and *T. pratense* (triangles), in dependence of AMF combination (CC = AMF-free / AMF-free, CI = AMF-free / *R. irregularis*, CM = AMF-free / *F. mosseae*, II= *R. irregularis* / *R. irregularis*, MI= *F. mosseae* / *R. irregularis*, MM = *F. mosseae* / *F. mosseae*).

Shoot-root ratio

Plantago lanceolata always had a significantly smaller shoot:root ratio than *T. pratense* (difference = –0.65, 95% CI: –1.2 to –0.2). Shoot:root ratios and the effect of AMF treatments on shoot:root ratio were dependent on the identity of the plant species ($F_{5, 83} = 9.57$, $p = <0.001$ for the interaction AMF treatment × species identity): AMF presence increased the shoot:root ratio in *Trifolium pratense* (due to greater shoot biomass) but not in *P. lanceolata*, in which the ratio was even decreased when *F. mosseae* was present on both root sides (due to greater root biomass).

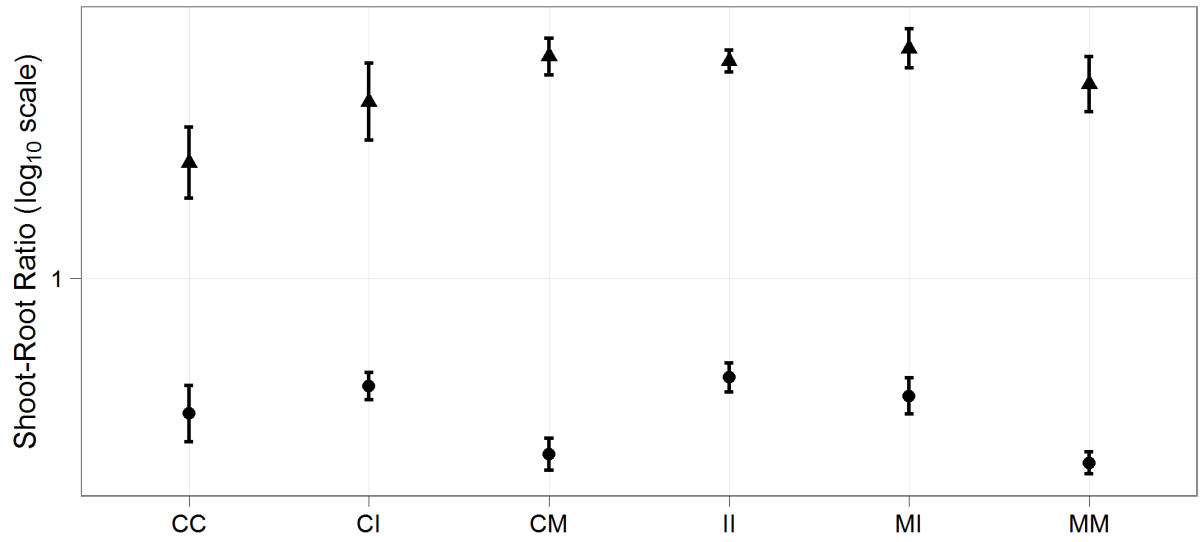

Figure 5. Shoot-Root ratio (mean ± 1 SE) for *P. lanceolata* (circles) and *T. pratense* (triangles), in dependence of AMF combination (CC = AMF-free / AMF-free, CI = AMF-free / *R. irregularis*, CM = AMF-free / *F. mosseae*, II= *R. irregularis* / *R. irregularis*, MI= *F. mosseae* / *R. irregularis*, MM = *F. mosseae* / *F. mosseae*).

^{33}P transferred to the plant shoot

We found no transfer of ^{33}P to the plant shoots in the absence of AMF in the hyphal compartment, which verified that our method was successful at achieving ^{33}P transport to the plant strictly through the AMF hyphae. *Rhizophagus irregularis* transferred 0.4% (95% CI: 0.2–0.7) of total ^{33}P added to the compartment more to the shoot than did *F. mosseae*, irrespective of plant species ($F_{1,30} = 10.1$, $p = 0.004$). However, *T. pratense* shoots[?] received 0.5% (95% CI: 0.1–1.0) of total ^{33}P added to the compartment more than did *P. lanceolata* ($F_{1,6} = 4.42$, $p = 0.08$). We found no evidence for significant differences in ^{33}P in the shoot due to the AMF identity of the opposite root side for either plant species ($F_{1,28} = 0.30$, $p = 0.59$ for the interaction between AMF treatments on the two sides).The results were similar for total plant P (data not shown). Taken together, this demonstrates greater ^{33}P transfer to shoots by *R. irregularis* than by *F. mosseae.* Also, more ^{33}P was acquired by *T. pratense* than by *P. lanceolata*.

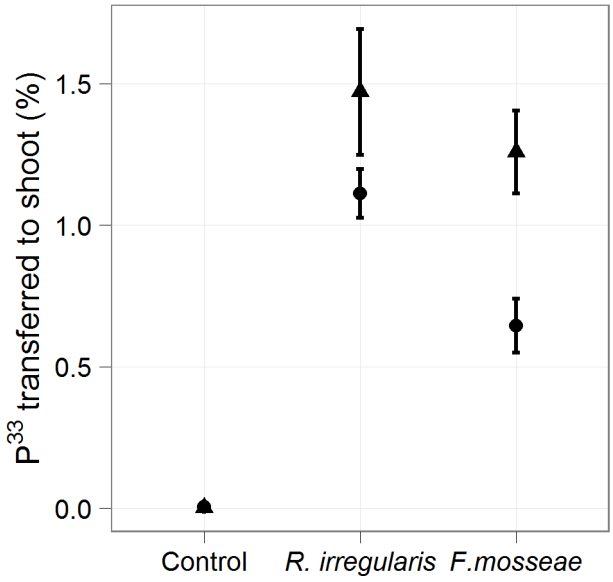

Figure 6. Percentage (mean ± 1 SE) of the total ^{33}P added to the compartment that was transferred by either of the two AMF to shoots of *P. lanceolata* (circles) and *T. pratense* (triangles).

AMF colonization

We found that *T. pratense* had on average 11.3% (95% CI: 1.1–23.4) higher AMF colonization than *P. lanceolata* ($F_{1,123}$=57.45, p<0.001). In *T. pratense*, infection rates did not depend on AMF identity (64.6%, 95% CI: 59.3–60.0 for *F. mosseae* and 65.7%, 95% CI: 60.5–70.8 for *R. irregularis*) whereas *P. lanceolata* had significantly lower infection from *F. mosseae* (39.8%, 95% CI: 33.1–46.5) than with *R. irregularis* (54.4%, 95% CI: 48.1–60.8; $F_{1,123}$=8.16, p<0.001 for the interaction plant species x AMF identity).

Partner selection by the plant?

Belowground plasticity: percentage of total root biomass allocated to each compartment

The two plant species varied in their response to the number of AMF present and the identity of the AMF on roots. *Trifolium pratense* always allocated more than 50% of the total root biomass to the root side with AMF present regardless of the AMF identity of that side when AMF was absent from the other side (*F. mosseae* = 73.3, 95% CI: 64.9–81.7 and *R.*

irregularis = 64.5, 95% CI: 56.1–72.8). However, if both root sides had AMF then allocation was nearly equal between sides regardless of the identity. Conversely, *P. lanceolata* only allocated significantly more than 50% root biomass to a side with the combination of *F. mosseae* on one side and no AMF on the opposite side (57%, 95% CI: 51.3–62.6). Therefore, *T. pratense* displayed larger plasticity in root biomass allocation than did *P. lanceolata*.

^{14}C allocated to roots (Bq per mg)

Trifolium pratense had at least 1.1 Bq mg^{-1} (difference between the control treatments of the two species) more ^{14}C concentration in roots than *P. lanceolata* ($F_{1,6}$=41.5, p = 0.0007). The two species responded differently to the presence of AMF. *Trifolium pratense* had more ^{14}C enriched roots in the presence of AMF while *P. lanceolata* had less ^{14}C concentration in presence of AMF ($F_{2,141}$=5.84, p=0.004 for the interaction plant species x treatment). Furthermore, *P. lanceolata* had significantly less ^{14}C-enriched roots regardless of the AMF identity. *Trifolium pratense* had more ^{14}C-enriched roots colonized by AMF than *P. lanceolata* but only significantly more in roots colonized by *R. irregularis* (1.1 Bq per mg, 95% CI: 0.2–2.0). However, both species had larger ^{14}C concentrations in roots colonized by *R. irregularis* than by *F. mosseae* (0.3 Bq per mg for *P. lanceolata* and 0.5 Bq per mg for *T. pratense*, $F_{2,141}$=2.9, p=0.06). We found no evidence for significant differences in ^{14}C concentration for either AMF species due to the AMF identity of the opposite root side for either plant species ($F_{2,99}$=0.46, p=0.63 for the interaction between treatments on the two sides). The results were furthermore similar to the ^{14}C losses by respiration (data not shown) and confirm greater allocation of ^{14}C to AMF by *T. pratense* than *P. lanceolata*.

^{14}C allocated to the hyphal compartment (Bq)

We found more ^{14}C in the hyphal compartments of *P. lanceolata* than T. *pratense* (37.0 Bq per comparment, 95% CI: 8.7–65.3) and the AMF species affected the amounts of

^{14}C in the hyphal compartments with higher values in compartments with *F. mosseae* than those with *R. irregularis* ($F_{1,91}$=18.74, p<0.0001). Moreover, we found reduced ^{14}C to the hyphal compartment with presence of the less cooperative *F. mosseae* when the choice of R. *irregularis* was offered in the opposite hyphal compartment (-33.8 Bq per compartment, 95% CI: –65.4 to –2.3).

Carbon costs per phosphorous transferred

We also calculated the ratio of labeled C in the two root-system halves (root compartment plus hyphal compartment) per labeled P in the shoot, which allowed us to perform a cost:benefit analysis for each plant–AMF combination. *Trifolium pratense* always had lower ratios than *P. lanceolata* regardless of the AMF species it was associated with ($F_{1,49}$=21.34, p<0.0001). Furthermore, *F. mosseae* resulted in significantly higher carbon costs than *R. irregularis* regardless of the plant species ($F_{1,49}$=9.46, p=0.003). These results allow us to rank the plant–AMF combinations from highest to lowest cost: (1) *P. lanceolata–F. mosseae*, (2) *P. lanceolata–R. irregularis*, (3) *T. pratense–F. mosseae* and (4) *T. pratense–R. irregularis*). We found no evidence for significant differences in carbon costs for either AMF species due to the AMF treatment of the opposite side for either plant species ($F_{2,49}$=1.32, p=0.28 for the interaction between treatments on the two sides).

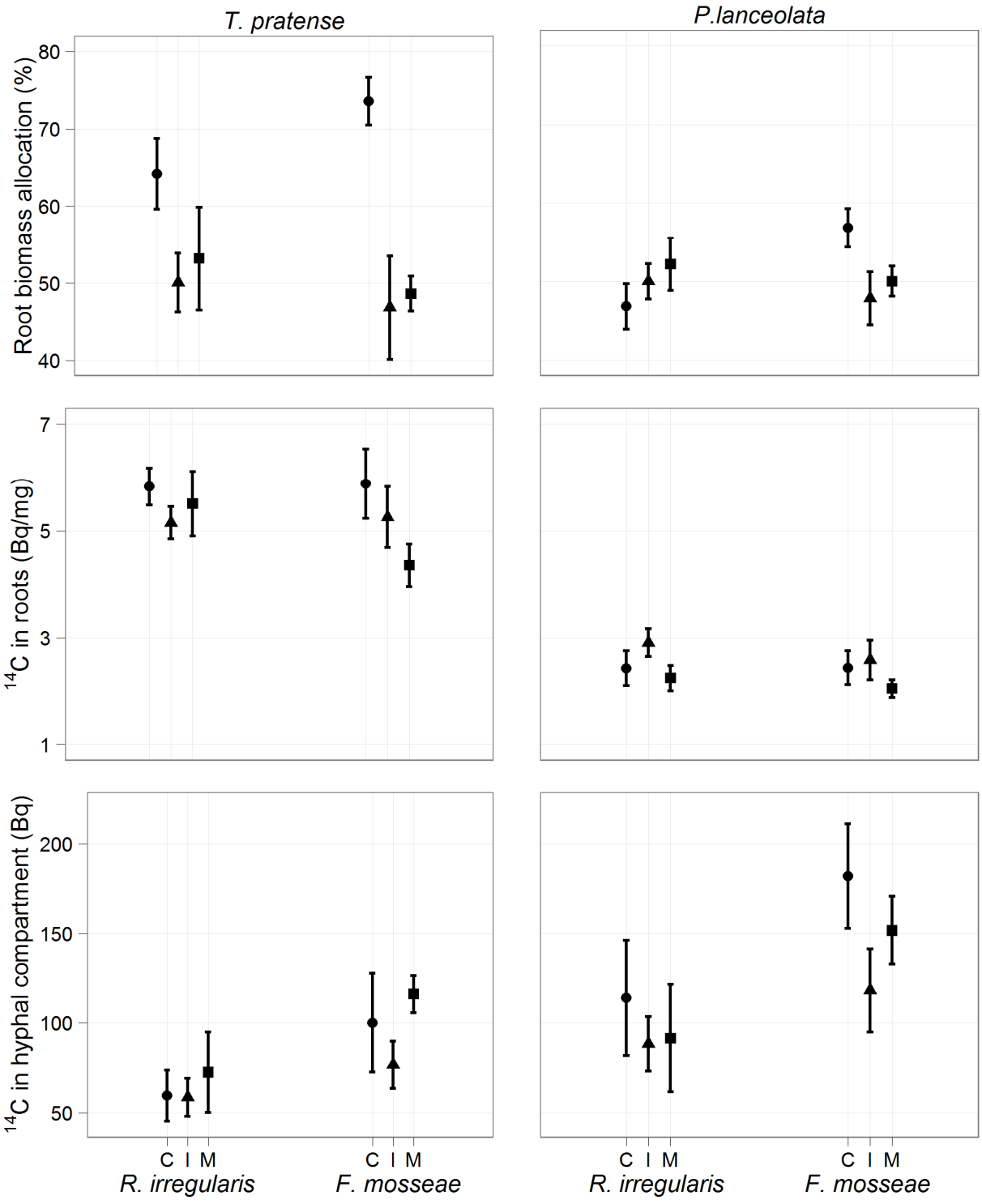

Figure 7. Root biomass on labeled side as percentage of total root biomass (top panels), ^{14}C allocated to roots on labeled side (middle panels) and ^{14}C allocated to the hyphal compartment on labeled side (bottom panels) as a function of AMF treatment on the labeled side for each treatment on the un-labeled side (Control: circles, *R. irregularis*: triangles and *F. mosseae*: squares). Points represent means with standard error bars. (C = AMF-free, I = *R. irregularis*, M = *F. mosseae*)

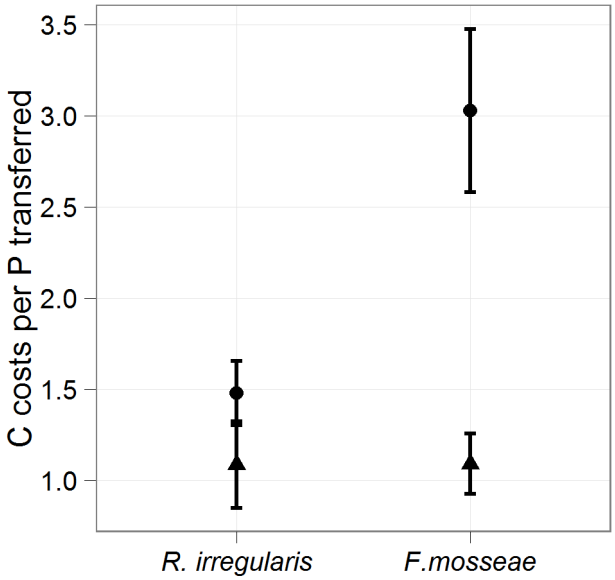

Figure 8. Carbon allocated to belowground (root + hyphal compartments) per unit of phosphorus transfer to the shoot (mean ± 1 SE) per AMF partner and plant species. Circles represent *P. lanceolata* and triangles represent *T. pratense*.

Discussion

In our study, we examined *in vivo* the nutrient exchange (carbon for phosphorus) in the plant–AMF association and the existence of preferential partner rewards using radioactive isotopes and compartmentalization in split-root systems, in which the roots of a single plant were divided into separate halves inoculated with different AMF species as partners. We used two plant and two AMF species to quantify the natural trade in the plant–AMF interaction for both more and less dependent plant species as well as for both more and less cooperative AMF species. As expected, we found a larger beneficial effect (enhanced plant biomass) of the presence of AMF for *T. pratense* than for *P. lanceolata* and a more mutualistic behavior of *R. irregularis* than of *F. mosseae*. These effects were reflected by a changed shoot:root ratios and larger amounts of AMF-provided phosphorus in the plants. In line with recent studies on AMF partner choice (Bever *et al.*, 2009; Kiers *et al.*, 2011), we found that roots infected by the more cooperative *R. irregularis* were more ^{14}C enriched than roots colonized by *F.*

mosseae and reduced ^{14}C allocation to hyphal compartments in presence of the less cooperative *F. mosseae* when the choice of *R. irregularis* was offered in the neighbor compartment. Moreover, in our study, we further found that the less cooperative *F. mosseae* seemed to be a better mutualist with *T. pratense* than with *P. lanceolata*, but the legume also allocated relatively more carbon (in terms of both relative root biomass and ^{14}C concentrations) to the AMF partners than did *P. lanceolata*. Additionally, the carbon costs per unit of phosphorous transferred to the plant were always lower for *T. pratense* than for *P. lanceolata* and especially when associating with *F. mosseae*.

These results help to explain why both plant species consistently allocated more carbon to the more cooperative AMF species (*R. irregularis*) and additionally demonstrate how the services provided by the plant (in terms of ^{14}C allocation) also affected the cooperative behavior of the AMF partners. Our results are similar to recent studies by Kiers *et al.* (2011) in which plants could perceive and reward the most cooperative AMF partner with more carbohydrates. Under experiments using *in vitro* root organs, they proved how AMF partners would support mutualism by increasing phosphorus transfer to those roots providing more carbohydrates. These types of responses are consistent with the evolutionary stability of the mutualistic plant–AMF interaction because partner control is bidirectional and comparable to an economic market, where partners with the best rate of exchange are preferentially rewarded (Schwartz & Hoeksema, 1998; Kiers *et al.*, 2011).

In addition, in our study we included two different plant species differing in their mycorrhizal dependencies to investigate *in vivo* whether higher quality services from the plant were better remunerated by the AMF. Hence, even when we found that both plant species, *P. lanceolata* and *T. pratense,* obtained more phosphorus and reduced carbon cost with the more cooperative AMF, *R. irregularis* (Fig. 6 & 8) *T. pratense* always obtained more phosphorus than *P. lanceolata*, especially from the "a priori" less mutualistic *F. mosseae*. This greater

remuneration was however consistent with a higher allocation of ^{14}C and relative root biomass of *T. pratense* to the AMF partner (except in the case of the soil from the hyphal compartment, but this fraction was comparatively much lower). Additionally, *T. pratense* had higher AMF colonization levels, which is usually associated with a higher level of resource exchange (Hodge & Fitter, 2010). Furthermore, the legume might have provided the AMF with additional services (e.g. improving nitrogen content); however, we did not specifically carry out tests for this.

Despite our knowledge of many aspects of the plant–AMF association which have the potential to influence the net effect of the association (e.g. identity of partners (Chapter 1), spatial structure (Chapter 2) or resource availability (Chapter 3)), more than half of the existing variation in plant growth still remains unidentified (Hoeksema *et al.*, 2010). In particular, analyses of the uptake and trade of carbon and soil nutrients and the reciprocal flux of resources between plants and AMF have been inadequately studied (Grman *et al.*, 2012).

Our study reveals how enhanced cooperation from both partners can improve the mutualism of the plant–AMF interaction, helping to justify its evolutionary persistence. Although more empirical evidence is necessary across a greater diversity of plant and AMF species and environments (e.g. nutrient availability), the indication of bidirectional control in the reciprocal flux of resources in our *in vivo* study, using two plant and two AMF species that differ in their mutualistic quality, suggests that these observations may be general (Bever, 2009). Because of the key role of the plant–AMF association in ecosystem function (Klironomos *et al.*, 2011; Wagg *et al.*, 2011a,b), understanding the mechanisms that determine the resource trade between plant and AMF partners is essential to evaluate the potential impact of the plant–AMF association at larger scales, such as the potential carbon sequestration by AMF in elevated CO_2 scenarios or the nutrient facilitation by AMF for ecosystem restoration.

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General Discussion

Intelligent behavior is an adaptive process that has evolved to enable organisms to deal with dynamic environmental conditions. Because intelligence is a complex behavior that implicates the whole organism and commonly seen as a fast response, the intelligent abilities of plants have largely been questioned (Trewavas, 2005). In fact, plants have no central nervous system with which to integrate decision-making when dealing with changes in their environment. However, plants are able to show phenotypic plasticity as a response to multiple abiotic (e.g. light, water, nutrients) and biotic (e.g. presence of competitors, pathogens, herbivores) environmental factors. A large part of this plasticity is a consequence of the need for active foraging for resources, but may have its origin in the ability of plants to internally detect, integrate and select the best choice.

Nutrient limitation is one of the most common challenges plants cope with during their lifespan. Plants have developed various strategies to maximize the acquisition of nutrients such as plasticity in root biomass allocation or association with symbionts such as the Arbuscular Mycorrhizal Fungi (AMF). In the latter case, plants invest their own photosynthesized carbon in a fungal partner in exchange for nutrients that otherwise would not be accessible to the plants. However, there might be some risks for the plants when associating with AMF. First, despite that the plant–AMF symbiosis is often considered mutualistic, the net effect on plant fitness ranges from mutualistic to parasitic (Kiers & Van Der Heijden, 2006) depending on the ecological conditions (e.g. nutrient availability) and plant–AMF combinations. Second, AMF can colonize several plant individuals at the same time and commonly form large mycorrhizal networks (Whitfield, 2007). In these networks, different individuals of plants are interconnected by mycorrhizal hyphae where nutrients and carbon can move from one plant to another (Selosse *et al.*, 2006). This creates many

opportunities for the exchange of resources that may lead to positive or negative interactions among plant and AMF species.

In these circumstances, theory suggests that selection should favour individuals that take more than they give ("cheaters"), but two closely interacting species of plant and AMF may evolve a mutualistic relationship if cheaters can be excluded (Egger, 2004; Kiers & Van Der Heijden, 2006). In fact, it is still unclear how the mutualism between plants and AMF is maintained. Preferential allocation to more cooperative partners and bidirectional control of the resource exchange (trade) have recently been suggested as the main probable processes to stabilize the cooperation in this ancient symbiosis (Bever *et al.*, 2009; Kiers *et al.*, 2011).

In my thesis, I examined the net outcome of the plant–AMF symbiosis and based on this I tested for signs of intelligent behavior by the plants. I investigated the existence of decision-making processes in plants growing in split-root systems with heterogeneous biotic (e.g. variation of AMF presence or identity) and abiotic (e.g. variation in nutrient availability) environments. I further explored the conditions of trade for both plant and AMF partners which determine the outcome of the plant–AMF symbiosis and tested for signs of preferential allocation and reciprocal rewards *in vivo*.

My results confirm that the beneficial effects of the AMF in plants largely depend on the identity of both plant and AMF. In the first chapter, I show how the same AMF species can have opposite directionality in the interactions depending on the plant identity, but also how the same plant species could have responses (e.g. total biomass) that range from negative to positive depending on AMF identity. From these results I selected the most suitable plant–AMF combinations for my next experiments where I further investigated and related plant decision-making with the plant–AMF symbiosis.

The following three chapters of my thesis deal at least in part with the existence of decision-making processes in plants. I clearly and recurrently found that plants preferentially allocated resources (in terms of root biomass and ^{14}C) to roots in the presence of AMF. The strength of the allocation was dependent on plant identity (Chapters 3 and 4), AMF identity (Chapters 2 and 4) and the increase of nutrient availability (Chapter 3). More interestingly, I found that plants could modify their resource allocation (again in terms of root biomass and ^{14}C) not only in response to AMF presence but also depending on AMF identity (Chapter 2 and 4). Thus, in heterogeneous AMF combinations, plants preferentially allocated biomass and ^{14}C to roots with the more beneficial AMF partners. However, this preferential allocation may depend on the experimental conditions in which plants are grown. I grew plants in nutrient-poor sandy soils. Under such resource-limited conditions, preferential allocation to the AMF is probably most likely to occur. Therefore, I suggest further studies including a gradient in nutrient content to allow calculating the strength of the preferential allocation to AMF under a range of nutrient availability.

To investigate whether plants detect and potentially integrate specific signals from AMF or whether they only respond to the increased nutrient supply created by the AMF, I applied different combinations of AMF and nutrient treatments to the two halves of split-root systems of test plants (Chapter 3). Plant responses were analyzed both at the level of the entire plant and at the level of the two halves of the root system to examine how plants integrate information. In homogeneous treatments, *T. pratense* was mostly and positively affected by AMF presence while *P. lanceolata* was equally and additively affected by AMF presence and nutrient availability for biomass and negatively affected by AMF presence regarding nitrogen contents.

In heterogeneous treatments, *P. lanceolata* showed positive integration for nutrient heterogeneity and, at low nutrient availability, for AMF heterogeneity, whereas *T. pratense*

showed no integration for nutrient heterogeneity and negative integration of AMF heterogeneity. Remarkably, there were correlative responses between the two halves of root systems of single plants exposed to spatial heterogeneity in AMF presence or nutrient availability, suggesting that plants can integrate signals coming from different parts. In particular, biomass allocation towards roots growing in either AMF patches or nutrient-rich patches was increased at the within-plant level, which was in contrast to the decreased allocation of biomass to roots when entire plants were growing in homogeneous AMF or nutrient conditions.

My study suggests that plants perceive AMF presence and nutrient availability as different signals and are able to show integrated responses accordingly, both at the level of the individual plant and of the root system. However, the ability to respond precisely to spatial environmental variability was greater in *P. lanceolata* than in *T. pratense* and I hypothesize that is due to greater plasticity in root foraging strategies of *P. lanceolata* and higher mycorrhizal dependency of *T. pratense.* The clear differential response between the two plant species demonstrates the importance of AMF affecting species coexistence (Van der Heijden *et al.*, 2003). Because plants with greater plasticity in root foraging strategies like *P. lanceolata* would have an advantage over species like *T. pratense* in patchy nutrient environments, I hypothesize that the presence of AMF could act as a stabilizing component by preventing competitive exclusion of plant species with inferior foraging abilities.

In the fourth chapter, I found strong evidence to support the hypotheses that the reciprocal reward between partners is a process that stabilizes cooperation in the plant–AMF symbiosis. I examined *in vivo* the exchange of resources between plants and AMF using two plant species differing in their mycorrhizal dependencies *(P. lanceolata* and *T.pratense)* and two different AMF isolates varying in their beneficial effects on plants (*Rhizophagus irregularis* and *Funneliformis mosseae*), which allowed me to use natural variation in the

plant–AMF trade. I found that both AMF partners were better-quality symbionts with *T. pratense* than with *P. lanceolata*, but the first plant species also allocated relatively more carbon to the AMF partners than did *P. lanceolata*.

Additionally, I proved that the carbon costs per unit of phosphorous transferred to the plant were always higher for *P. lanceolata* than for *T. pratense* and especially when associating with the less cooperative *F. mosseae*. I hypothesize that *T. pratense*, a legume with high mycorrhizal dependency, may have evolved a closer and more mutualistic relationship with AMF (Egger, 2004) and therefore the reciprocal rewards and the net outcome of the symbiosis was more beneficial for both symbionts. Because in my study I used two plant species and two AMF partners that differ in their symbiont quality, the indication of bidirectional control in the reciprocal flux of resources in both systems suggests that these observations may be generalized to a wider range of systems (Bever *et al.*, 2009). However, to fully understand the conditions of trade and confirm its importance in stabilizing the existence of the mutualism in the AMF symbiosis, future studies should be extended to more plant–AMF combinations and environments (e.g. nutrient availability).

I used a relatively artificial model system for my experiments. The major deviation from a natural system was the spatial structure created by the compartments of the split-root systems, the presence of only two AMF and the absence of multiple plant–AMF interactions. Because spatial structure can alter the outcomes of mutualism (Doebeli & Knowlton, 1998; Bever & Simms, 2000; Hoeksema & Kummel, 2003; Verbruggen *et al.*, 2012), further work should try to resemble more natural scenarios, where spatial structure is not as rigidly imposed as in our split-root systems. Also, in plant–AMF associations, host plants are typically infected by multiple AMF species (Vandenkoornhuyse *et al.*, 2002; Scheublin TR, Ridgway KP, Young JPW, 2004) and can furthermore be connected through complex mycorrhizal networks (Whitfield, 2007). In my studies, because of the specific questions

addressed in this thesis and methodological limitations, I only used two AMF isolates per plant species and did not allow mycorrhizal networks to form. Nevertheless, the results described here must be due to processes that can occur in natural systems and that may underpin plant decision-making and the mutualism in the plant–AMF symbiosis.

Future effort should be made in achieving a more natural system. Recently developed genetic techniques such as quantitative PCR could be included in upcoming work on the topic. As shown by Wagg et al. 2011, this technique allows detecting the abundance of different AMF species in a single root and therefore estimating the relative contribution to plant fitness of each AMF without the need of physical separation. The combination of quantitative PCR with the inclusion of AMF networks in the system could clarify the nutrient exchange among sysmbionts, the relative importance of each member of the network and the possible alterations of the resource exchange in conditions in which multiple interactions among plant species and AMF are co-occurring.

Conclusion

In this thesis, I have found ample evidence that support decision-making processes in plants and the existence of preferential allocation and reciprocal rewards as responsible mechanisms for the persistence of mutualism in the plant–AMF symbiosis. These results are consistent with recent studies about the bidirectional control in the plant–AMF associations and confirm its existence using plants *in vivo*. However, the conditions of the resource exchange in natural environments are still unknown and efforts should be made in achieving study systems that more closely resemble nature.

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Curriculum Vitae

Personal

Surname	ARGÜELLO GARCÍA
First name	Alicia
Date of Birth	12 December 1984
Nationality	Spain

Education

1998 – 2002	IES Legio VII, León, Spain
2002 – 2007	B.Sc. (Long Cycle Degree) Environmental Sciences with Major in Environmental Technology, University of León, Spain
2006 – 2007	Erasmus Student, Vrije Universiteit of Amsterdam, The Netherlands
2007 – 2009	M.Sc. Ecology, Department of Animal Ecology and Systems Ecology, Vrije Universiteit of Amsterdam, The Netherlands. Advisors: Prof. Marcel van der Heijden, Susanne de Bruin and Dr. Peter van Bodegom
2009 – 2013	Ph.D. Natural Science, Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland, Advisor: Prof. Bernhard Schmid

Posters

Arguello A, Wiemken A, Schmid B. (2010). Plant-decision making and AMF symbiosis: Can plants really choose the most beneficial partner? Symposium of the Zurich-Basel Plant Science Center. Basel (Switzerland).

Presentations

Arguello A, Schmid B. (2012). Are plants able to integrate spatially separated signals from AMF presence and nutrient availability? 3rd Swiss Arbuscular Mycorrhiza Meeting (SAM-2012), University of Fribourg (Switzerland).

Arguello A, Niklaus PA, Schmid B. (2011). Plant-decision making and AMF symbiosis: Can plants really choose the most beneficial partner? 2nd PSC-Syngenta Symposium at Syngenta Crop Protection Research Center in Stein Säckingen (Switzerland).